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The role of the hypoxic microenvironment on the macrophage-tumor cell interplay

Flávia Oliveira Martins

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Dissertação de Mestrado apresentada à
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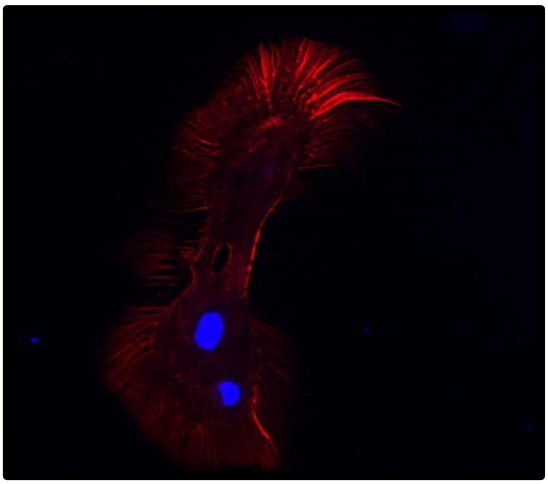

The role of the hypoxic microenvironment on the macrophage-tumor cell interplay

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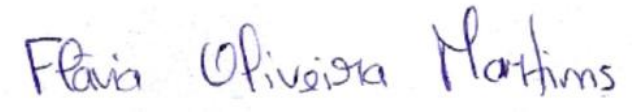

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Resumo

A hipoxia é uma característica comum dos tumores sólidos. Os tumores sólidos tem um microambiente que é composto por células tumorais, matriz extracelular, e células do estroma e imunes que cooperam e afetam umas às outras. Uma das células mais abundantes no microambiente tumoral são os macrófagos que podem atuar como supressores (macrófagos do tipo M1) ou promotores (macrófagos do tipo M2) da atividade tumoral, dependendo do estímulo a que estão expostos.

Para compreendermos como é que o microambiente tumoral hipóxico afeta a relação entre macrófagos e células tumorais, estabelecemos um sistema de co-cultura com uma linha de cancro colorectal (RKO) e macrófagos humanos, em condições de normoxia (20%O_2) ou de hipoxia (1%O_2). Macrófagos são derivados de monócitos, isolados de dadores de sangue, e foram cultivados sozinhos ou com células tumorais RKO, nas condições anteriormente descritas. Além disso, células tumorais RKO também foram cultivadas sozinhas, nas mesmas condições. Depois da co-cultura, ambas as populações foram caracterizadas por diversas técnicas.

Primeiro, expressão de mRNA da anidrase carbónica IX (*CAIX*) foi avaliada em células tumorais de RKO para confirmar que as células responderam à hipoxia. De seguida foram caracterizados por diversas técnicas, a funcionalidade e o perfil inflamatório dos macrófagos. Em relação às células tumorais, as atividades celulares associadas com invasão também foram avaliadas.

A caracterização dos macrófagos revelou que tanto células tumorais como hipoxia, individualmente ou em conjunto, induzem um fenótipo misto em macrófagos, com características de ambos macrófagos do tipo M1 ou M2. Hipoxia, por si só, induz alterações na expressão de marcadores de polarização, na atividade fagocitária e na expressão de genes relacionados com o metabolismo. No entanto, a presença de células tumorais é mais poderosa do que a hipoxia na modulação da área celular dos macrófagos, da atividade metabólica e do metabolismo de lactato e glucose. Todavia, a combinação da hipoxia e da co-cultura é capaz de potenciar a fosforilação das proteínas NF-kB e IkBα.

Em relação ao efeito da hipoxia e dos macrófagos nas células tumorais, nós descobrimos que diversos mecanismos estão envolvidos na resposta a estes estímulos. Nós descobrimos, nas condições analisadas, que a hipoxia tem um efeito mais poderoso que a co-cultura tendo em conta a expressão de genes relacionados com o metabolismo e de alguns genes associados com EMT, assim como na fosforilação das proteínas Src e ERK. Nós também observamos que, em contraste, a co-cultura com macrófagos é

mais poderosa que a hipoxia na modulação da expressão de *CD47*, no metabolismo de lactato e glucose e na expressão de outros genes relacionados com EMT. No entanto, a combinação de hipoxia e co-cultura com macrófagos tem um efeito potenciado na expressão de *CD47*, capacidade de invasão e migração das células tumorais e na fosforilação das proteínas Src e ERK.

O modelo utilizado neste trabalho, um sistema de co-cultura indireto, é um modelo simples mas robusto para investigar a interação entre macrófagos e células tumorais, num ambiente em normoxia ou hipoxia, *in vitro*. É uma ferramenta importante para compreender como é que as células tumorais e os macrófagos comunicam uns com os outros, e como é que respondem a hipoxia, permitindo a caracterização dos mecanismos envolvidos nesta resposta. No geral, nós consideramos que o conhecimento obtido neste trabalho fornece informação importante que pode contribuir para abordagens terapêuticas mais eficientes, quer na re-educação de macrófagos associados a tumores, ou utilizando as células tumorais como alvo, evitando a sua invasão.

Palavras-chave: Hipoxia; macrófagos; cancro colorectal; microambiente tumoral; invasão células tumorais; co-culturas

Abstract

Hypoxia is a common feature of solid tumors. Solid tumors microenvironment is composed by cancer cells, extracellular matrix (ECM), and stromal and immune cells that cooperate and affect each other. One of the most abundant cells at the tumor microenvironment are macrophages that can act as tumor suppressors (M1-like) or promotors (M2-like), depending on the stimuli they are exposed to.

In order to understand how hypoxic tumor microenvironment affects the interplay between macrophages and tumor cells we established a co-culture system with a CRC cell line (RKO) and human macrophages, in normoxia (20%O_2) and hypoxia (1%O_2). Macrophages are monocyte-derived, isolated from healthy blood donors, and were cultured alone or with RKO cancer cells, under the conditions previously described. Additionally, RKO cancer cells was also cultured alone, under the same conditions. After co-culture, both populations were characterized by several techniques.

First, mRNA expression of anhydrase carbonic IX (*CAIX*) was evaluated in RKO cancer cells to confirm that cells respond to hypoxia. Following, macrophage functionality and inflammatory profile were characterized by through several techniques. Concerning cancer cells, invasion-associated cellular activities were also evaluated.

Macrophage characterization revealed that either cancer cells or hypoxia, individually or together, induced a mixed phenotype in macrophages, with both M1 and M2-like characteristics. Hypoxia by itself induce alterations in the expression of polarization markers, in the phagocytic activity and the expression of metabolism-related genes. However, the presence of cancer cells, is powerful than hypoxia in the modulation of macrophage cell area, metabolic activity and lactate/glucose metabolism. Nevertheless the combination of hypoxia and co-culture is able to potentiate the phosphorylation of NF-kB and IkBα proteins.

Concerning the effect of hypoxia and macrophages on cancer cells, we found that several mechanisms are involved in cancer cell response to these stimuli. We found in the conditions analyzed that hypoxia have a more powerful effect than the co-culture regarding the expression of metabolism-related genes and of some EMT-associated genes, as well as in Src and ERK proteins phosphorylation. We also observed that, in contrast, the co-culture with macrophages is powerful than hypoxia in modulating *CD47* expression, lactate/ glucose metabolism and the expression of others EMT-associated genes. However the combination of hypoxia and co-culture with macrophages have a potentiated effect on *CD47* expression, invasion and migration capacity of cancer cells and phosphorylation of Src and ERK proteins.

The model used in this work, an *in vitro* indirect co-culture system is a simple model to investigate the interaction between macrophages and cancer cells, under normoxic or hypoxic environment. It is an important tool to understand how cancer cells and macrophages connect to each other and how they respond to hypoxia, allowing to characterize the mechanisms involved in this response. Overall, we consider that the knowledge obtained with this work provide important information that might contribute to more efficient therapeutic approaches, either in re-educating tumor-associated macrophages, or targeting cancer cells, avoiding their invasion.

Keywords: Hypoxia; macrophages; colorectal cancer; tumor microenvironment, cancer cell invasion, co-cultures

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List of Abbreviations

APC – Adenomatous Polyposis Coli

APC – Allophycocyanin

ATCC – American Type Culture Collection

BSA – Bovine Serum Albumin

CAFs – Cancer Associated Fibroblasts

CAIX – Carbonic Anhydrase IX

CCL – C-C Chemokine Ligand

CCR – C-C Chemokine Receptor

CD – Cluster of Differentiation

CIMP – CpG Island Methylator Phenotype

CIN – Chromosomal Instability

CRC – Colorectal Cancer

CXCL – C-X-C Chemokine Ligand

DAPI – 4', 6-Diamidino-2-Phenylindole

DCs – Dendritic Cells

ECM – Extracellular Matrix

EGF – Epidermal Growth Factor

EKA – Electro Kinetic Analyzer

ELISA – Enzyme-linked Immunosorbent Assay

EMT – Epithelial-Mesenchymal Transition

ERK – Extracellular signal-regulated kinase

FACs – Fluorescence-Activated Cell Sorting

FAP – Familial Adenomatous Polyposis

FAP – Fibroblast Activated Protein

FBS – Fetal Bovine Serum

FGFs – Fibroblast Growth Factors

FIH – Factor Inhibiting HIF

FITC – Fluorescein Isothiocyanate

FSP – Fibroblast Specific Protein

GLUT-1 – Glucose Transporter 1

HIF – Hypoxia Inducible Factor

HRE – Hypoxia Response Elements

IFN-γ – Interferon - gamma

IL – Interleukin

LDHA – Lactate Dehydrogenase A

LOX – Lysyl Oxidase

LPA – Lysophosphatidic Acid

LPS – Lipopolysaccharide

M-CSF – Macrophage Colony-Stimulating Factor

MDSCs – Myeloid-derived Suppressor Cells

MFI – Mean Fluorescence Intensity

MHC – Major Histocompatibility Complex

MMPs – Matrix Metalloproteinases

MSI – Microsatellite Instability

NF2 – Neuron Glial Antigen

NK – Natural Killers

P4H – Prolyl 4-Hydoxylase

PBMCs – Peripheral Blood Mononuclear Cells

PBS – Phosphate-Buffered Saline

PDGFR – Platelet Derived Growth Factor Receptor

PDGFs – Platelet Derived Growth Factors

PE - Phycoerythrin

PFA – Paraformaldehyde

PHDs – Prolyl-4-Hidroxilase Domains

pVHL – Von Hippel-Lindau protein

qRT-PCR – Quantitative Real-Time Polymerase Chain Reaction

R.P.M – Rotations Per Minute

ROI – Reactive Oxygen Intermediates

RT – Room Temperature

SDS – Sodium Dodecyl Sulfate

SIRP1α - Signal Regulatory Protein 1 alpha

SMA - Smooth Muscle Actin

TAMs – Tumor Associated Macrophages

TGF-β – Transforming Growth Factor–beta

TME – Tumor Microenvironment

TNF-α – Tumor Necrosis Factor-alpha

TNM – Tumor/Node/Metastasis

VEGF – Vascular Endothelial Growth Factor

Introduction

In the past twenty-five years major advances have been made in the cancer research field, which generated a complex network of knowledge, establishing cancer as a complex and dynamic disease, involving changes in the genome. Cancer is characterized by major hallmarks, meaning that, cancer cells are a manifestation of essential alterations in the cell physiology, such as: i) self-reliance in proliferation signals; ii) insensitivity to growth suppressors; iii) resisting programmed cell death (apoptosis); iv) unlimited replicative potential; v) continuous angiogenesis; vi) inducing tissue invasion and metastasis and reprogramming of energy metabolism; vii) avoiding immune destruction; viii) tumor-promoting inflammation; ix) genome instability and mutation; x) deregulating cellular energetics (Hanahan & Weinberg 2011)

1. The tumor microenvironment

Tumor microenvironment is not just composed by a mass of malignant cells, but is a complex and heterogeneous system composed of numerous cells and structures. Interactions between the tumor and the host non-transformed cells create a unique environment, the tumor microenvironment (TME), which is determinant for cancer progression (Joyce & Pollard 2009). In solid tumors, which are organ-like structures, the tumor microenvironment is composed, besides malignant cells, by stroma fibroblasts, endothelial and infiltrated immune cells, embedded within an extracellular matrix (ECM) (Fig. 1) (Trédan *et al*. 2007; Whiteside 2008; Balkwill *et al*. 2012; McDonald *et al*. 2016), enriched in soluble secreted factors (Hui & Chen 2015). Some examples of soluble growth factors present at the tumor microenvironment are vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and epidermal growth factor (EGF), which are regulators of cell migration, adhesion, and angiogenesis, modulating cancer progression (Guan 2015).

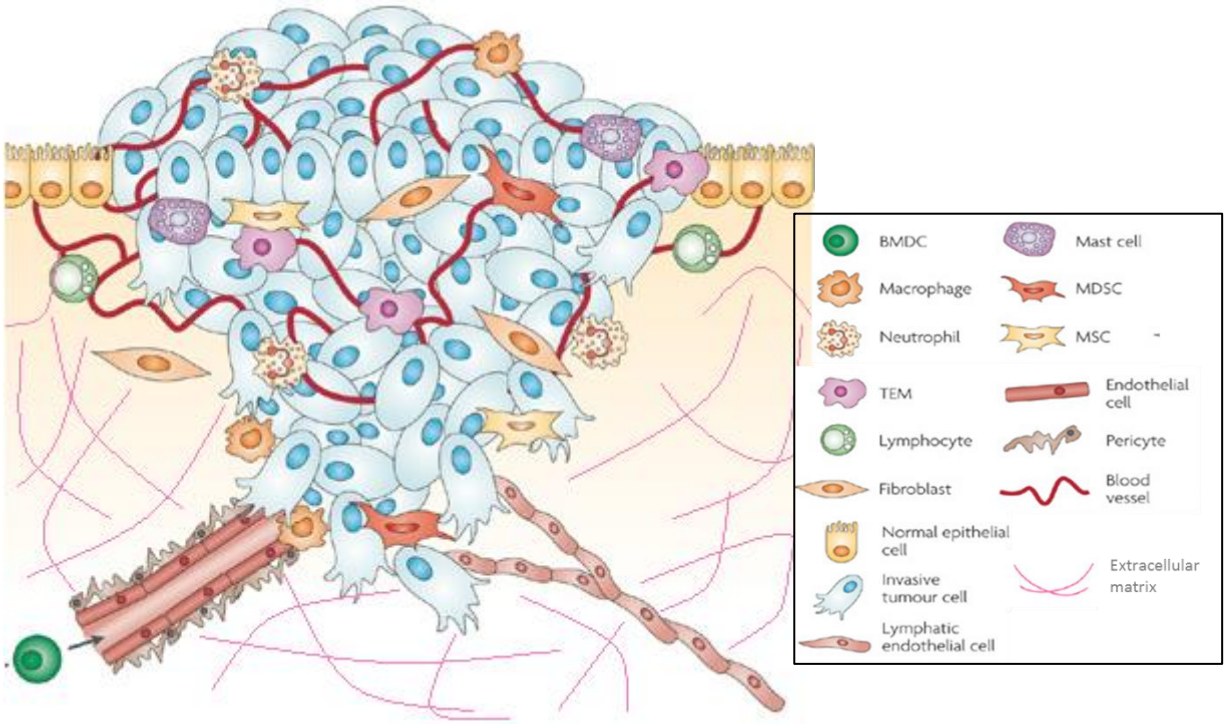


Figure 1. The tumor microenvironment. Solid tumors are complex microenvironments composed of both cancer and non-malignant cells, supported by the extracellular matrix. The cellular component of the tumor microenvironment includes fibroblasts, endothelial cells, pericytes, and a variety of immune cells, namely macrophages, neutrophils, lymphocytes, mast cells, myeloid-derived suppressor cells (MDSC), mesenchymal stem cells (MSC), bone-marrow derived dendritic cells (BMDC) and TIE-2 expressing monocytes (TEM). Adapted from Joyce & Pollard 2009 and Xu, Farach-Carson, and Jia 2014.

1.1 Cellular components

Besides malignant cells, the cellular component of the tumor microenvironment is composed by a variety of non-tumor cells, which include fibroblasts, adipocytes, endothelial and immune cells. None of these cells of the stroma are themselves malignant, but they acquire altered functions and an abnormal phenotype due to interactions with cancer cells (Li *et al*. 2007; Balkwill *et al*. 2012). Moreover, the stromal cells can contribute to tumor cell survival, proliferation, invasion and metastasis, by the production of soluble growth factors and chemokines (Dranoff 2004; Li *et al*. 2007; Balkwill *et al.* 2012) that release to the tumor microenvironment.

i. Fibroblasts

Fibroblasts, which in normal tissues are associated with tissue stroma, are the main producers of ECM components, as collagen and fibronectin, and actively contribute to the formation of the basement membrane (Li *et al*. 2007). Additionally, fibroblasts are associated with tissues wound healing and, when activated, exhibit an increased proliferation and alterations in their secretory capacity (Li *et al*. 2007; Balkwill *et al*. 2012). When associated with cancer, fibroblasts are named cancer-associated fibroblasts

(CAFs), and are the main cellular component of the tumor stroma. CAFs are associated with the loss of CD34 and the enhanced expression of α-smooth muscle actin (α-SMA), fibroblast specific protein (FSP), platelet derived growth factor receptor-α and β (PDGFR-α, PDGFR-β), neuron glial antigen 2 (NG2) and of fibroblast activated protein (FAP). Nevertheless, CAFs share with normal non-activated fibroblasts the expression of several stromal markers as fibronectin, vimentin, stromelysin or matrix metalloproteinase (MMP) 3 and prolyl 4-hydroxylase (P4H) (Tomasek *et al*. 2002). Activation of stromal fibroblasts are associated with factors secreted by tumor cells, such as transforming growth factor-beta (TGF-β), interleukin-6 (IL-6), IL-1β and lysophosphatidic acid (LPA) that have the ability to transform precancerous fibroblasts into CAFs; and fibroblast growth factors (Mueller & Fusenig 2004). Moreover, CAFs also secret growth factors, cytokines and MMPs, which are associated with the induction of epithelial-mesenchymal transition (EMT), growth and migration of tumor cells. (Li *et al*. 2007; Balkwill *et al.* 2012).

ii. Adipocytes

Adipocytes are a cell type able to store excess of energy in the form of lipids. These cells are considered endocrine cells and are known to produce growth factors, hormones and adipokines. Secretion of adipokines and other factors by adipocytes has been associated with promotion of cancer cell proliferation, invasive potential, survival and angiogenesis (Rajala & Scherer 2003). Additionally, adipocytes have been also associated with the recruitment of tumor cells and associated with tumor growth of intra-abdominal tumors that metastasize to omentum. Adipokines secretion aid in recruitment of tumor cells, and fatty acids provided by adipocytes work as source of energy for cancer cells, promoting tumor growth (Amemori *et al*. 2007; Balkwill *et al*. 2012).

iii. Endothelial cells

Endothelial cells and their associated pericytes are stimulated by soluble factors present in the tumor microenvironment, such as VEGFs, FGFs, platelet-derived growth factors (PDGFs) and chemokines. The stimulation of vascular endothelial cells is required during the development of a complex vascular network (neovascularization), that is necessary for tumor growth (Li *et al*. 2007; Carmeliet & Jain 2011). VEGF is the predominant angiogenic factor in TME and is secreted by tumor cells, fibroblasts and immune cells in the stroma. VEGF is a pro-angiogenic factor, meaning that it stimulates angiogenesis, and therefore the formation of abnormal vessels, with irregular shape, and fragile. The abnormal vessels increase the interstitial fluid pressure and originate irregular blood flow, which promotes hypoxia that in turn increases VEGF, facilitating tumor cell invasion and metastasis (Jain 2005; Carmeliet & Jain 2011).

iv. Immune cells

Notably, almost all immune cell populations can be found within the tumor microenvironment: those intervening in adaptive immunity, as T lymphocytes, dendritic cells (DC), and occasionally B cells, and effectors of innate immunity, such as macrophages, polymorphonuclear leukocytes and natural killer (NK) cells (Li *et al*. 2007; Whiteside 2008). The immune cells present within the tumor microenvironment are involved in the anti-tumor immune response, aiming tumor elimination. Leukocytes are initially recruited into the tumor surrounding in response to several cytokines and chemokines secreted by tumor cells (Coussens & Werb 2002). Cancer cells surface or release antigens are captured and processed by the recruited immature DCs, which become activated. Additionally, some pro-inflammatory cytokines released by tumor cells that are dying potentiated DCs recruitment and activation. DCs will present the antigens captured on major histocompatibility complex (MHC) molecules to B and T cells, originating a T cell response against the presented tumor-specific antigens (Chen & Mellman 2013). Activation of B cells will contribute to the establishment of a memory response but also to the activation and recruitment of innate immune cells, such as macrophages. On the other hand, DCs-activated T cells will migrate from the lymph nodes back to the tumor microenvironment and specifically recognize, bind and kill cancer cells, which releases additional tumor-associated antigens, recruiting more T cells (Visser *et al*. 2006). However, this anticancer immune response is not always effective, and sometimes the tumor antigens are not detected, and therefore, cancer cells escape to immune surveillance. Moreover, the cancer cells can subvert the immune response, tuning pro- into anti-inflammatory cells with pro-tumor properties and that by releasing extracellular proteases, pro-angiogenic factors and growth factors and chemokines (Coussens & Werb 2002; Visser *et al*. 2006; Chen & Mellman 2013), may promote tumor immune escape, cancer cell invasion and metastasis.

1.2 Extracellular matrix

The extracellular matrix functions as a structural scaffold for the tumor microenvironment cells and is composed of approximately 300 proteins involved in the regulation of tissue homeostasis, organ development, inflammation and disease (Gilkes & Wirtz 2014; Chanmee *et al.* 2014). Although diverse on its composition, the ECM comprises fibrous proteins (collagens and elastin), adhesive proteins (fibronectin and laminin), glycosaminoglycan/proteoglycans and a multitude of growth factors (such as angiogenic factors and chemokines) that are locally secreted and arrested *(*Balkwill *et al*. 2012; Gilkes & Wirtz 2014). The different components of the ECM can be divided in two main forms: the basement membrane and the interstitial matrix. Basement

membrane is produced by epithelial, endothelial and stromal cells and is considered a specialized ECM, more compact and less porous than the interstitial matrix. Additionally, the basement membrane is mainly composed by collagen type IV, laminins and fibronectin and proteoglycans. By its turn, the interstitial matrix is composed mainly by stromal cells and is enriched in fibrillary collagens, proteoglycans and glycoproteins (Lu *et al.* 2012).

ECM regulates cell polarity, differentiation, survival, proliferation, migration and has also a role in tumor initiation and progression. ECM remodeling is regulated by the expression of enzymes such as MMPs, which can alter ECM dynamics, leading to its disorganization and changes of its essential properties (Lu *et al*. 2012; Martin *et al.* 2016). The role of abnormal ECM dynamics is very well studied, and documented as a hallmark of cancer (Lu *et al*. 2012). Abnormal changes in ECM composition and organization potentiate oncogenic effects in various signaling pathways, which may lead to tumor initiation and invasion (Pinto *et al*. 2017). Dense ECM (collagen density) and fibrosis is described as players in tumor initiation in breast and liver cancers, respectively. Additionally, alterations in biochemical properties, such as production of more ECM components, as hyaluronic acid, and ECM stiffness are also related with worse prognosis (Lu *et al*. 2012; Martin *et al*. 2016).

2. Macrophages

The monocytes that move from the blood stream to the peripheral tissues, in response to local chemotactic and growth factors, pro-inflammatory cytokines and microbial compounds, differentiate into macrophages (Tacke *et al.* 2006), which consist of a heterogeneous population of phagocytic and antigen-presenting cells that play a fundamental role in the immune system.

Macrophages have decisive functions in both innate and acquired immunity, being their main functions the immediate defense against foreign pathogens and the modulation of adaptive immune responses through antigen processing and presentation (Sica *et al*. 2008; Fraternale *et al*. 2015). Macrophages cooperate with T and B cells, through cell to cell interaction and by the release of several molecules as cytokines, chemokines, enzymes, arachidonic acid metabolites and oxygen and nitrogen reactive species (ROS and NOS). Beyond this role on the modulation of the immune system, macrophages are also involved in several functions, such as tissue remodeling, thrombosis (Sica *et al.* 2008) and homeostasis (Duque & Descoteaux 2014).

2.1 Origin of macrophages

Tissue macrophages are a heterogeneous population that can be divided by their origin as: the tissue-resident macrophages, which develop at the embryonic stage, and the monocyte-derived macrophages, that differentiate from bone marrow-derived monocytes. The resident macrophages, at the embryonic stage are mainly derived from cells from the yolk sac or the fetal liver, originating the microglia or Langerhans cells, respectively. Additionally, the monocyte progenitors colonize different tissues, such as liver, brain, skin and lung and differentiate in resident macrophages, as Kupffer cell, microglia, Langerhans and alveolar macrophages, able to self-maintain through life. On the other hand, macrophages can also differentiate from bone-marrow derived monocytes, which migrate into different tissues in response to specific stimuli. (Davies *et al*. 2013; Franklin & Li 2016).

2.2 Macrophage polarization

One of the main features of macrophages is their high plasticity, meaning that their phenotype can change depending on the local environment signals (Leblond *et al*. 2016; Fraternale *et al*. 2015; Egners *et al*. 2016), which makes them interesting therapeutic targets. Depending on the stimuli, macrophages polarize into different subtypes, being the extremes of polarization the M1-like phenotype or classically activated, and the M2-like phenotype or alternatively activated (Cassetta *et al*. 2011; Leblond *et al*. 2016). This nomenclature (M1/M2) is derived from the Th1 and Th2 cytokines that are associated with these macrophage phenotypes (Fraternale *et al*. 2015), and were first proposed by Mills in 2000 (Mills *et al*. 2000).

The pro-inflammatory M1 phenotype is considered as anti-tumoral, whereas the anti-inflammatory M2 phenotype is considered as pro-tumoral (Noy & Pollard 2014; Fraternale *et al*. 2015; McDonald *et al*. 2016). The pro-inflammatory M1 macrophages are generally polarized in response to interferon-gamma (IFN-γ), lipopolysaccharides (LPS) and cytokines, as tumor necrosis factor-alpha (TNF-α), and secrete hight levels of interleukins (IL)-1, IL-12, IL-23, IL-6, nitric oxide, reactive oxygen intermediates (ROI) and low IL-10 (Mantovani *et al*. 2004; Sica & Mantovani 2012; Zhang *et al*. 2013; Cole *et al*. 2014; Murray *et al*. 2014; Leblond *et al*. 2016). Additionally, M1 macrophages are potent effector cells able to kill microorganisms and control tumor growth, and express specific surface receptors, namely cluster of differentiation (CD) 80, CD86 and also MHC class I and class II molecules, which are required for the presentation of tumor-specific antigens (Mantovani *et al*. 2002; Mantovani *et al.* 2004). On their turn, the anti-inflammatory M2 macrophages are usually polarized by IL-4, IL-13, IL-10, glucocorticoid hormones, and produce high levels of IL-10, IL-2 and IL-8, and low levels of IL-12, IL-23

and IL-6 (Mantovani *et al*. 2004; Hao *et al*. 2012; Sica & Mantovani 2012; Zhang *et al*. 2013; Cole *et al*. 2014; Martinez & Gordon 2014; Murray *et al*. 2014; Fraternale *et al*. 2015), and display high levels of the mannose receptor (CD206) and of the scavenger receptor (CD163) (Mantovani *et al*. 2004).

Due to the diversity of macrophage functions, the initially proposed M1/M2 phenotype system is now considered as an oversimplification (Braster *et al*. 2015). Nowadays, macrophage polarization is denoted as a spectrum (Fig. 2), with possible mixed phenotypes, containing features from M1 and M2 phenotypes. In general, and acording to Cardoso *et al*, M1 macrophages present a more elongated morphology than M2 macrophages (Cardoso *et al*. 2015). Nonetheless, analysis of the macrophage morphology is not enough to have a full characterization of their polarization profile. For a complete macrophage profile it is necessary to evaluate their gene expression profile, protein surface markers, as well as analysis of secreted inflammatory citokines and chemokines. Consequently, the M2-like phenotype was subdivided in different subpopulations, based on their distinct stimulation and gene expression profiles: the M2a, stimulated by IL-4 and IL-13, the M2b, activated by immunocomplexes such as IL-1 receptor ligands and LPS, M2c, induced by IL-10, TGF-β and glucocorticoids, and the most recent M2d, in response to IL-6 and adenosine. *(*Mantovani *et al.* 2004*;* Sica *et al*. 2006; Metchnikoff & Prize 2015). In generally, M2a are associated with parasites killing, M2b macrophages with immunoregulatory functions, M2c macrophages with immune responses and tissue remodeling and M2d macrophages have some features of tumor-associated macrophages (TAMs), like promoting tumor growth and angiogenesis *(*Mantovani *et al.* 2004; Sica *et al.* 2006; Fraternale *et al.* 2015; Weagel *et al*. 2015).

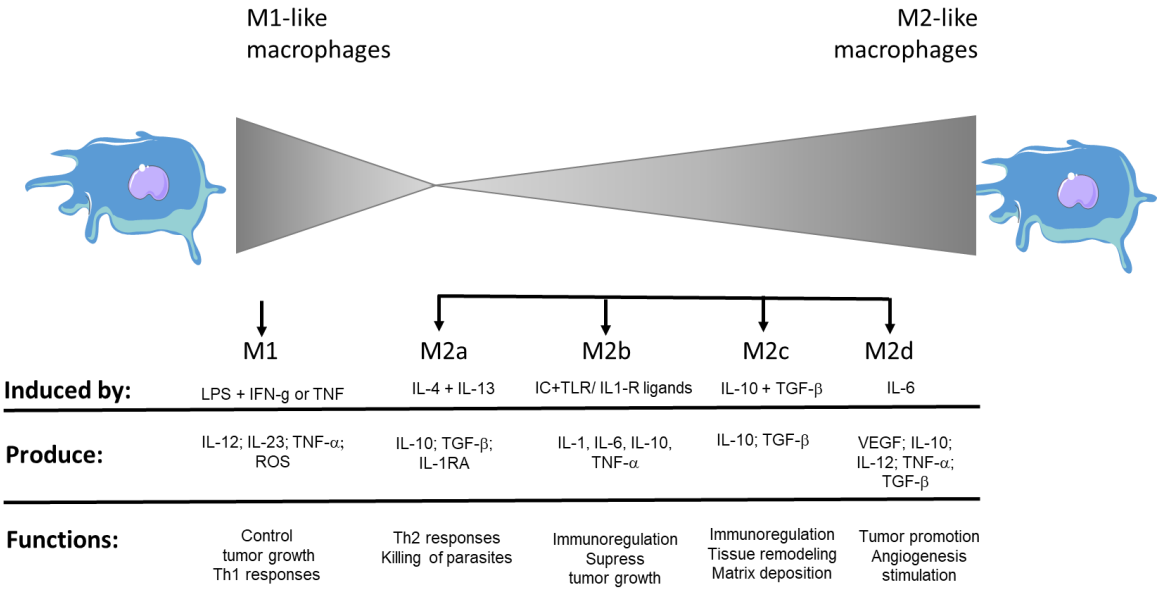


Figure 2. Spectrum of macrophage polarization. Classification of macrophage polarization as a continuum spectrum between M1-like and M2-like macrophages. Several stimuli are identify to induce M1 or different M2 macrophage subtypes, determining the surface receptors that they express, the factors that they secrete and their function. Adapted from Weagel *et al*. 2015

2.3 Tumor-associated macrophages

Monocytes are dynamically recruited into tumors, where they differentiate into tumor-associated macrophages (TAMs) (Mantovani *et al*. 2006; Li *et al*. 2007; Whiteside 2008; Joyce & Pollard 2009; Fraternale *et al*. 2015; McDonald *et al*. 2016; Chanmee *et al*. 2014). These TAMs exhibit a high degree of phenotypic plasticity (McDonald *et al*. 2016) and represent frequently the most abundant immune cell population at solid tumors microenvironment. TAMs are recruited to tumor tissue mainly by cytokines, chemokines and growth factors produced by cells within the tumor microenvironment, both tumor and stromal cells. Many factors have been reported to promote macrophage recruitment, such as tumor-derived C-C Motif Chemokine Ligand 2 (CCL2), CCL3, CCL4, CCL5, CCL7, CCL8, C-X-C Motif Chemokine Ligand 12 (CXCL12), macrophage colony stimulating factor (M-CSF), VEGF, and IL-1. (Hao *et al*. 2012).

Generally, TAMs are referred as belonging to the M2-like population, with M2-related functions, including scavenging and tissue-repair properties, promotion of proteolysis and angiogenesis (Sica *et al*. 2008; Fraternale *et al*. 2015) and the ability to stimulate cancer cell migration, invasion and metastasis (Condeelis & Pollard 2006; Cardoso *et al*. 2015). These macrophage functions are associated with secretion of growth factors as EGF, TGF-β and VEGF, cytokines/chemokines and enzymes involved in ECM remodeling, such as MMPs (Sica *et al*. 2008). TAMs have been associated with tumor microenvironment factors that differentiate macrophages towards an M2-like phenotype, such as the anti-inflammatory IL-10. It was demonstrated by our group that IL-10-polarized macrophages are more efficient in promoting *in vitro* angigonesis and cancer cell invasion (Cardoso *et al*. 2015). Additonally, TAMs seem to be preferentially attracted to and accumulated in necrotic and hypoxic regions of the tumor (Li *et al*. 2007; Shen *et al*. 2013; Fraternale *et al*. 2015), although can also be found at highly vascularized regions (Ohno *et al*. 2003; Coffelt *et al*. 2009).

According to several studies in distinct cancer types, high levels of TAMs infiltration are associated with poor prognosis, reduced overall survival and therapy resistance (Mantovani *et al*. 2006; Zhang *et al*. 2013). Nevertheless, in colon carcinomas the role of TAMs is controversial, since some works described the increase of macrophage infiltration as a good prognosis marker (Gordon & Martinez 2010) while others report the opposite (Pollard 2004). Our group is currently mapping the distribution of distinct

macrophage subpopulations along normal mucosa, tumor center and invasive front, aiming to elucidate their contribution for colon cancer prognosis.

3. Hypoxia: an hallmark of tumor progression

Hypoxia, usually defined as ≤ 2% O_2, is a common feature of solid tumors, and is commonly described as the imbalance between oxygen demand and supply *(Leblond et al*. 2016). The tumor microenvironment is frequently hypoxic due to the rapid proliferation of tumor cells and insufficient blood supply (Leblond *et al*. 2016; McDonald *et al*. 2016).

3.1 Role of hypoxia in tumor progression

Hypoxia has been correlated with tumor aggressiveness, described to contribute to malignant progression, local invasion, distant metastasis, and resistance to therapy (Mantovani *et al*. 2006; Li *et al*. 2007). Cancer invasion into adjacent tissues, the first step of the metastization process, is a complex and dynamic process, dependent on cancer cell intrinsic properties, as migration and ability to reorganize and degrade underlying ECM components (Friedl & Alexander 2011), and extrinsic factors, as the ECM composition and stiffness, hypoxia, extracellular vesicles, and the action of other cells from the microenvironment (Condeelis & Pollard 2006; Gligorijevic *et al*. 2014). Invasion is the result of the detachment of cells from the primary tumor as result of alterations on cytoskeleton organization, and on cell-matrix and cell-cell adhesion with the involvement of proteolytic enzymes, such as MMPs (Friedl & Alexander 2011). Invasion may also involve the transition from an epithelial-like to a mesenchymal-like phenotype (EMT), which is a feature of most invasive cells, and is often characterized by downregulation of the cell-cell adhesion molecule E-cadherin, upregulation of the type III intermediate filament vimentin, and lose of other epithelial characteristics (Clark & Vignjevic 2015).

Hypoxia is responsible for several cellular modifications such as metabolic alterations, and expression of enzymes that interfere with the ECM, such as lysyl oxidase (LOX), a collagen and elastin cross-linking enzyme, and MMPs (Erler *et al*. 2006; Gilkes *et al*. 2014). Besides, hypoxia is also associated with the activation of several signaling pathways, which lead to enhanced angiogenesis, inhibition of apoptosis, upregulation of growth factors as TGF-β and EGF, and promotion of cancer cell migration, proteolysis and invasion proteins (Mantovani *et al*. 2006).

3.2 HIFs and mechanism of hypoxia sensing

Hypoxia condition induction will result in the activation of hypoxia inducible factors (HIFs), which are cellular transcription factors involved in the response to environmental stress (Li *et al*. 2007), and whose activation leads to genetic reprogramming of both cancer cells and macrophages (Murdoch and Lewis 2005; Zhang *et al*. 2014). HIF is a heterodimeric complex, composed by α (with three isoforms: 1α, 2α and 3α) and β subunits. While the β subunit is constitutively expressed, the stability of the α subunit depends on oxygen tension. The isoforms have differential expression profiles and gene targets, being the HIF-1α expression ubiquitous while the HIF-2α expression is more restricted (Wiesener *et al*. 2002). Under normoxia, HIF-1α is subject to post-translational modifications, and is constantly inactivated by ubiquitination and proteossomal degradation. Notably, Prolyl-4-Hydroxylases (PHDs), the factor-inhibiting HIF (FIH) and the Von Hippel-Lindau tumor-suppressor protein (pVHL) control the repression of HIF-1α under normal oxygen levels. The modification required for degradation is hydroxylation, which is regulated by PHDs and occurs on proline residues. This hydroxylation allows pVHL binding, targeting HIF-1α for degradation. In response to hypoxia, PHDs are inactivated and unable to induce HIF-1α hydroxylation. As consequence, HIF-VHL-mediated ubiquitination is inhibited and HIF is not degraded, being rapidly stabilized and translocate to the nucleus (Fig. 3). Once at nucleus, HIF-1α binds to HIF-1β to form a heterodimer, and induces the activation of promotors of various target genes containing hypoxia response elements (HRE), resulting on their transcription (Kenneth & Rocha 2008; Carnero & Lleonart 2016; D'Ignazio *et al*. 2016; Bertout *et al*. 2008; Durán *et al*. 2013). Its target genes, such as carbonic anhydrase IX (CAIX) and c-Met, are involved in cellular and physiological alterations, as energy metabolism and cell migration, respectively (Schofield & Ratcliffe 2004).

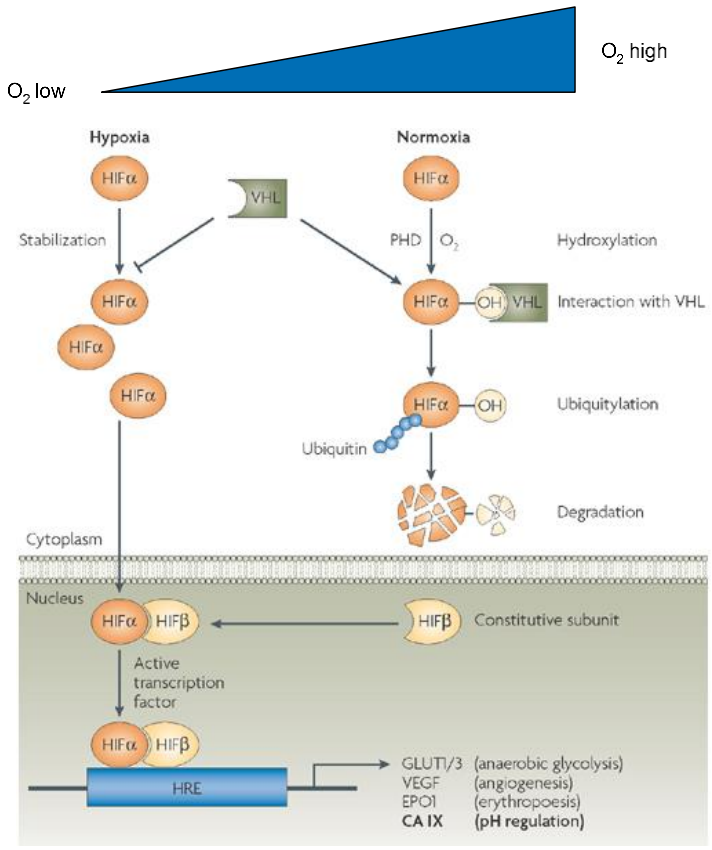


Figure 3. Mechanism of hypoxia-induced gene expression mediated by the HIF transcription. At normal oxygen levels (normoxia), prolyl-4-hydroxylase (PHD) hydroxylates hypoxia inducible factor-α (HIFα). The von Hippel-Lindau protein (VHL) binds hydroxylated HIFα and targets it for degradation by the ubiquitin–proteasome system. Under hypoxia, HIFα is not hydroxylated. Non-hydroxylated HIFα is stabilized and accumulates. After translocation to the nucleus, HIFα dimerizes with the HIFβ constitutive subunit to form an active transcription factor. The HIF transcription factor then binds to hypoxia response element (HRE) in target genes and activates their transcription. Adapted from Supuran 2008.

3.3 Hypoxia influence on macrophages

As discussed in a previous section, macrophages are extremely plastic cells, changing their phenotype in response to different stimuli. One example of such change with impact on gene expression is hypoxia. Some studies addressing the influence of hypoxia in macrophages have been described to upregulate the activity of certain transcription factors, such as HIF-1 and HIF-2 (Fang *et al*. 2009). Additionally to the upregulation of HIFs, other studies also reported alterations in the expression of genes related with macrophage survival, tissue revascularization, matrix remodeling and recruitment of more inflammatory cells (Lewis & Murdoch 2005; Fang *et al*. 2009). Some examples of genes whose expression is altered by hypoxia are glucose transporter 1 (GLUT-1), VEGF, TNF-α and MMP7 (Burke *et al*. 2003; Fang *et al*. 2009). Notably, it has been reported that macrophages accumulate in tumor hypoxic areas of ovarian, colorectal, prostate and breast cancers (Lewis & Murdoch 2005). Macrophages

accumulation in these hypoxic areas is explained by the release of chemoattractants factors secreted by the tumor and surrounding cells in response to the reduced oxygen concentrations and to the necrotic debris existent in these areas (Tazzyman *et al*. 2014). The influence of hypoxia in macrophage polarization has been another field of interest in the past years, and some studies report that the hypoxic tumor microenvironment polarizes macrophages into a more M2-like phenotype (Mantovani *et al*. 2002; Leblond *et al*. 2016). However, a recent study reported the opposite, and stated that hypoxia amplifies the pro-inflammatory profile of M1 macrophages and reprograms the M2 macrophages towards a more pro-inflammatory state (Varesio *et al*. 2016).

4. Colorectal cancer (CRC)

Colorectal cancer (CRC) is a major cause of morbidity and mortality all over the world (3rd most common), and one of the most prevalent cancers in both males and females, representing 10% and 9.2% of the total number of cases, respectively (Cappellani *et al*. 2013; Braster *et al*. 2015). This type of cancer is also referred as colon or rectal cancer, depending if the cancer starts in the colon or in the rectum, and these cancers are normally discussed together due to they features in common. Notably, colorectal cancer survival and prognosis is higly dependent upon the stage of disease at diagnosis (Haggar & Boushey 2009).

4.1 Epidemiology and risk factors

According to several epidemiological studies, the rates of CRC incidence and mortality vary across the distinct regions of the world (Cotter 2013), with a higher incidence in North America, Europe, Australia and New Zealand (Raskov *et al*. 2014). CRC is generally classified into two types: sporadic and associated with familial predisposition. Sporadic colorectal cancers are associated with somatic mutations linked mainly to environmental causes, and account for 70% of all CRC cases (Kim *et al*. 2013; Tarraga Lopez *et al*. 2014; Braster *et al*. 2015). Some patients are predisposed to develop CRC, due to hereditary diseases, such as familial adenomatous polyposis (FAP), hereditary non-polyposis CRC and ulcerative colitis, but these represents only about 30% of whole CRC cases (Jasperson *et al*. 2010; De Rosa *et al*. 2015). Example of risk factors associated with CRC development are: age, hereditary factors, environmental lifestyle-related risk factors as diet, smoking, obesity (Haggar & Boushey 2009), and inflammatory conditions of the digestive tract (Bishehsari *et al*. 2014; Siva Kumar *et al*. 2015). Additionally, higher CRC rates have been reported among populations that adopted Western-style diets (Kim *et al.* 2013; Bishehsari *et al.* 2014).

4.2 Carcinogenesis

Colon polyps are very common and most are benign (Tannapfel *et al*. 2010), but in some cases, if the polyps are not removed, cells can continue to proliferate and may progress into CRC, with ability to invade and metastasize (Tannapfel *et al*. 2010; Cappellani *et al*. 2013). Colorectal cancer can arise from one or a combination of three different mechanisms, such as chromosomal instability (CIN), CpG island methylator phenotype (CIMP) and microsatellite instability (MSI). Commonly, it is assumed, in the chormosomal instability pathway, the existence of a cascade of events that underlie the transition from adenoma to carcinoma. Therefore, mutations may affect the adenomatous polyposis coli (APC) tumor supressor gene, resulting in a early adenoma. Another alteration known to occur in the adenoma-carcinoma sequence is the activating mutation of the oncogene *KRAS,* which takes place on a late adenoma. The stage of adenocarcinoma is considered when occurs the functional inactivation of another tumor supressor gene, the *TP53* (Leslie *et al*. 2002; Armaghany *et al*. 2012) (Fig. 4). In the other hand, colorectal cancer may also originate from CpG island methylator phenotype, which is characterized by promoter hypermethylation of various tumor supressor genes, or from microsatellite instability, which predispose to genetic alterations (Tariq & Ghias 2016).

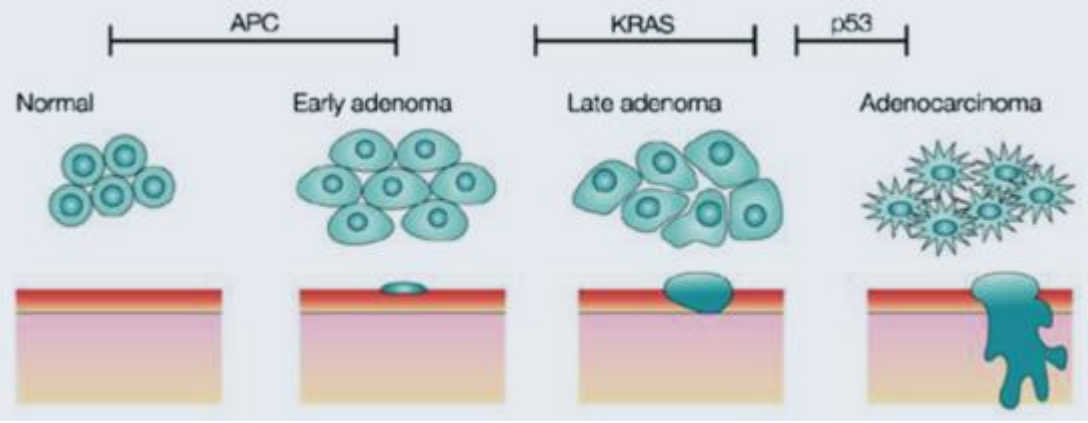


Figure 4. Adenoma-carcinoma cascade. Progression from normal epithelium through adenoma to colorectal carcinoma is characterized by accumulated abnormalities of particular genes. The initial step, resulting in an early adenoma is the loss of adenomatous polyposis coli (APC). Large adenomas acquire mutations in the oncogene K-*ras*, and the adenocarcinoma is characterized by the function inactivation of *p53.*Adapted from Armaghany *et al.* 2012.

4.3 Classifications and staging

Colorectal cancer staging is made according to the tumor/node/metastases (TNM) system specified by The American Joint Committee on Cancer (AJCC). The stages of colon cancer can be divided in Primary Tumor, regional lymph nodes infiltration and distant metastasis. Within these classifications, new divisions occur regarding the stage of disease in the specific site. Therefore, some additional information can be

assessed, as if tumor has invaded, the number of lymph nodes affect by metastasis and how much organs were affect by metastasis, which is important to classify the Colon Cancer stage (NCCN Clinical Practice Guidelines in Oncology: Colon Cancer).

4.4 Diagnosis and therapeutics of CRC

CRC is generally a cancer with a slow progression, implying that its early detection is possible, through several available screening methods, such as rectal exame, colonoscopy and fecal blood test (Delavari *et al.* 2014). If diagnosed early, most of CRC cases would benefit from surgical treatment (Pawa *et al*. 2011). However, when diagnosed, approximately 20% of the patients are already in a metastatic state. At this stage, the disease is considered incurable for most patients, and the best approach is then palliative care (Lucas *et al.* 2011), although some patients who have limited involvement of distant organs (liver and/or lung) can be cured with surgery (Goldberg *et al*. 1998; De Rosa *et al*. 2015). Therefore early diagnosis is of most importance and the development of more efficient therapeutic approaches is one of the researchers ultimate goals. If the tumor is completely removed, no further therapies are needeed, however, when CRC is already in a metastatic state, additional treatment can be required. For metastatic CRC the frequent treatment is palliative chemotherapy, which can improve survival, reduce symptoms and improve life quality (Cunningham *et al*. 2010).

5. Aims of this thesis

Our group has previously described that macrophages may modulate cancer cell activities, promoting cancer cells invasion, and elucidated the underlying molecular mechanisms. However, these studies were established under normoxia, not considering the effect of hypoxic conditions, abundant at the tumor microenvironment. The role of hypoxia in tumor progression has been analyzed by some authors as well as its effect on macrophages. However, how hypoxic impacts on the macrophage-cancer cell crosstalk remains unknown. Therefore, considering the relevance of hypoxia at the tumor microenvironment, the main goal of this thesis is to assess how hypoxia influences the macrophage-cancer cell crosstalk, and the cancer cell invasive phenotype, as a hallmark of malignancy, aggressiveness and poor prognosis, using CRC as a model. For this purpose, monocultures and co-cultures of macrophages and colon cancer cells were established under normoxia or hypoxia. The material collected was used to characterize macrophage and cancer cell properties, but also to assess the impact of hypoxia on macrophage-cancer cell crosstalk.

The effect of hypoxia and of cancer cells on macrophage polarization was assessed through the extensive analysis of macrophage pro- and anti-inflammatory markers.

Additionally, macrophage metabolic activity, cytoskeleton organization, phagocytic and proteolytic activities, and signaling pathways alterations were also analyzed. Furthermore, we evaluated the effect of hypoxia on macrophage-mediated cancer cell invasion, migration and proteolysis, as well as the impact of macrophages and hypoxia on cancer cell metabolic activity, expression of epithelial-mesenchymal transition related genes and modifications of invasion-associated signaling pathways.

Overall, with this project we expect to clarify how hypoxia influences the crosstalk between macrophages and colorectal cancer cells, in the cancer progression, elucidating the underlying molecular mechanisms which could open the way to new and more efficient therapeutics approaches.

Materials and Methods

Cancer cell culture

RKO cell line, derived from a colon carcinoma was purchased at American Type Culture Collection (ATCC). Cells were maintained at 37 ºC and 5% CO_2 humidified-atmosphere in RPMI 1640 medium with GlutaMax (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Biowest) + 100 UmL^{-1} penicillin and 100 μgmL^{-1} streptomycin (Invitrogen) (considered here as complete RPMI medium). This cell line was chosen due to its high response to macrophages demonstrated by our group in previous studies.

Human monocyte isolation and macrophage differentiation

Human monocytes were isolated from buffy coats of healthy blood donors, provided by Hospital São João. The blood from buffy coats was centrifuged at 1200 *g* for 20 min at room temperature (RT). The peripheral blood mononuclear cells (PBMCs), in a whitish layer, were collected and incubated with RosetteSep Human Monocyte Enrichment Cocktail (StemCell Technologies) for 20 minutes under rotation. This mixture was then diluted (1:1) with phosphate buffered saline (PBS) and 2% FBS (Biowest), added cautiously over Ficol-Histopaque 1077 (Sigma) and centrifuged at 1200 *g* (brake off) for 20-30 min at room temperature. The intermediated layer was collected, washed in PBS and centrifuged three times at 700 rotations per minute (r.p.m.) for 17 min.
Cells were resuspended in complete RPMI medium and $1.2x10^6$ macrophages were plated in 6-well culture plates with glass coverslips. Macrophages were differentiated for 10 days, and the cell culture medium renewed at day 7 post-isolation. The 10 days of differentiation included 7 days in the presence of macrophage colony-stimulating factor (M-CSF, ImmunoTools 50 $ngmL^{-1}$) and 3 days in absence of M-CSF. At the 10^{th} day, indirect co-cultures with cancer cells were performed.

Establishment of macrophage-cancer cell indirect co-cultures

Ten days after monocyte isolation, $1x10^5$ RKO cells were plated in 6 well-plate permeable transwell inserts (PET inserts with 1µm pore, Corning), and placed on top of macrophage cultures (Fig. 5). Inserts allowed the exchange of soluble factors between macrophages and cancer cells, avoiding cancer cells to cross from the top to the lower compartment. Co-cultures between macrophages and cancer cells were maintained in complete RPMI medium for 3 days (time usually used to macrophage polarization) in conditions of normoxia (20% O_2) or hypoxia (1% O_2). As control, macrophage and cancer cell monocultures were also performed. After 3 days of co-culture biological material was collected.

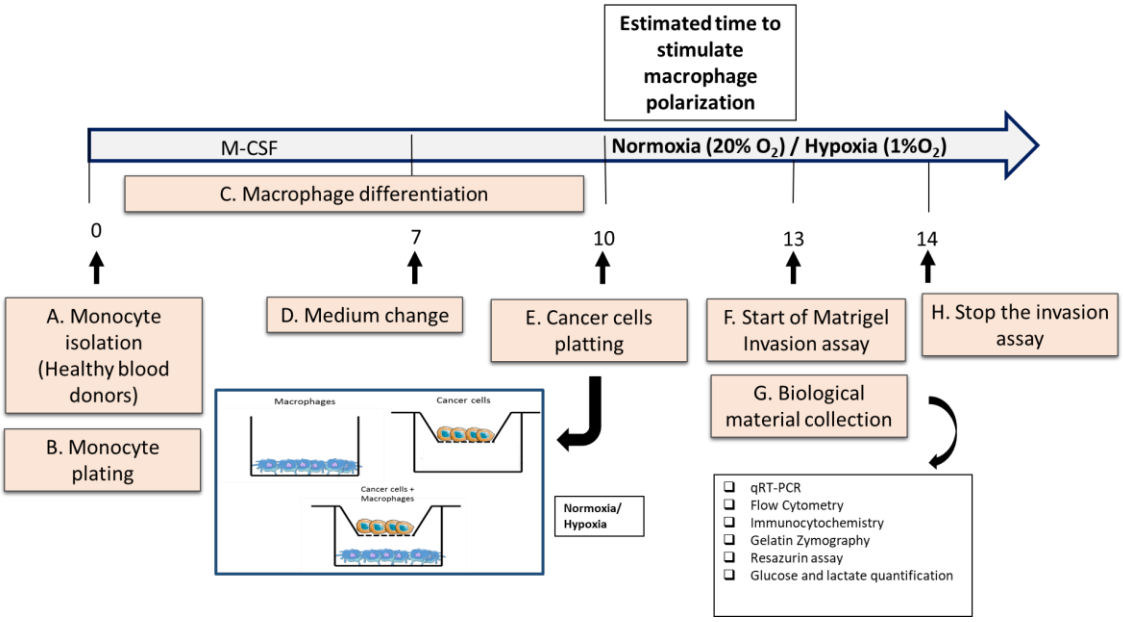


Figure 5. Schematic overview of the methodology used in this work. Monocytes were isolated from buffy coats from healthy blood donors, provided by Hospital São João. Monocytes were plated ($1.2*10^6$) on culture plates with RPMI medium with GlutaMax supplemented with 10% FBS, PenStrep and M-CSF for 7 days, and differentiated for 10 days. Cell culture media were renewed after 7 days, and the monocytes were left differentiating for more 3 days with RPMI complete medium, without M-CSF. Ten days after monocyte isolation, RKO cancer cells ($1*10^5$ cells/insert) were plated in transwell inserts and placed on top of macrophage cultures. Co-cultures were maintained in complete RPMI medium, under normoxia (20% O_2) or hypoxia (1% O_2) for 3 days. Macrophages and cancer cell monocultures were also prepared. After 3 days of co-culture, biological materials were collected and Matrigel Invasion assays were initiated.

Macrophage polarization

After ten days of macrophage culture, macrophages were stimulated for 3 days with 10 $ngmL^{-1}$ LPS or 10 $ngmL^{-1}$ IL-10 towards a pro- (M1-like) or anti-inflammatory (M2-like) phenotype, respectively.

Resazurin assay

The metabolic activity of macrophages and cancer cells, either cultured alone or in co-culture, was measured by resazurin reduction assay. Resazurin is a cell permeable redox indicator that can be used to monitor the metabolic activity of viable cells. Viable cells with active metabolism can reduce resazurin into the resorufin product, which is pink and fluorescent and can be quantified using a microplate fluorometer. After 3 days of co-culture, resazurin redox dye (0.01 $mgmL^{-1}$, Sigma-Aldrich) was added (1/10 of the total volume of culture medium) to cell culture wells (macrophages) or to transwell cell culture inserts (cancer cells), which were then incubated for 4h at 37ºC and 5% CO_2. Fluorescence intensity was measured (excitation 530 nm, emisson 590 nm) using the multi-mode microplate reader Synergy Mix (BioTek).

Invasion assay

The cell invasive phenotype were assessed using Matrigel-coated invasion inserts of 8µm pore-size filters (Corning) to mimic the basement membrane, using macrophages as invasive stimuli. Prior to experiment, filters were re-hydrated in RPMI medium for 1h at 37ºC. Confluent RKO cells were detached by trypsinization and re-suspended in complete RPMI medium, counted and $2.5x10^4$ cells were seeded on Matrigel-coated filter. Macrophages were plated in the lower compartment. Cells were incubated for 24h at 37ºC under normoxic and hypoxic conditions. The inserts were washed with PBS and fixed with 4% Paraformaldehyde. The non-invading cells, present on the top of the insert, were removed with a cotton swab. Cells that invaded were mounted in Vectashield Mounting Medium with DAPI (Vectashiel, Vector Laboratories) for nuclei staining, and visualized through a Leica DM2000 fluorescence microscope (Leica Microsystems).

Conditioned media processing

Conditioned media collected from monocultures and co-cultures of cancer cells and macrophages, exposed or not to a hypoxic environment, were centrifuged at 6000 rpm for 3 min. The supernatant was then transferred to a new Eppendorf and frozen at -20ºC.

Gelatin zymography

Gelatin zymography was performed to determine the activity of the matrix metalloproteinases MMP-2 and MMP-9. Protein concentration in conditioned medium was determined using the DCProtein Assay kit (BioRad) and 10 µg of protein were mixed with sample buffer [10% sodium dodecyl sulfate (SDS), 4% sucrose and 0.03% bromophenol blue in 0.5M Tris HCl, pH 6.8] and media were separated on 10% polyacrylamide zymogram gels with 0.1% gelatin (Sigma-Aldrich) as a substrate. After electrophoresis, gels were incubated in 2% Triton X-100 (Sigma) in deionized water for protein renaturation, during 30 minutes at room temperature. Subsequently, gels were washed with deionized water and incubated overnight with agitation, at 37ºC in MMP substrate buffer [50 mM Tris-HCl, pH 7.5; 10 mM $CaCl_2$]. After, gels were stained with Coomassie blue solution (Sigma-Aldrich) for 20 minutes and further incubated with destaining solution [20% methanol; 10% acetic acid], until the adequate resolution was obtained. MMPs activity was observed as white proteolytic areas against the blue background.

Sample preparation for Western Blot

Cells lysates were prepared to perform Western Blot. Cancer cells and macrophages were detached with trypsin (Gibco) or accutase (BD Biosciences), respectively, and scrapped, and centrifuged at 1200 r.p.m. for 5 min at RT. After centrifugation, supernatants were discarded, and added cold Rippa buffer [50 mM Tris HCl pH=7.5; 1% NonidetP (NP)-40; 150 mM NaCl and 2mM EDTA] with proteases/phosphatases inhibitors [10 mM NaF; 200mM phenylmethylsulfonyl fluoride (PMSF); 1 $mgmL^{-1}$ aprotinin and leupeptin; 50mM Na4VO3 and 50 $mgmL^{-1}Na_4P_2O_7$] was added to the pellets. The mixture of cells with lysis buffer was then placed on ice for 30 min, and centrifuged again at 13300 r.p.m. for 10 min at 4ºC, and supernatants collected. Protein concentration was determined using the DCProtein assay kit (BioRad) and 20 µg were mixed with Laemmli sample buffer [0.5 M Tris-HCl pH 6.8, 9.2g SDS, 40mL Glycerol, 5% β-mercaptoethanol, 5% bromophenol blue] and boiled for 6 min at 95ºC for protein denaturation.

Western blot

Samples were loaded on a 10% or 12.5% SDS-polyacrylamide gel and run at 100V. Following electrophoresis, gels were transferred to nitrocellulose membranes (GE Healthcare) for 2h at 100V. Membranes were then incubated with Ponceau (Sigma) solution until bands of proteins were visualized. Subsequently membranes were blocked with 5% non-fat powder milk in PBS + 0.1% Tween-20 (PBS-T 0.1%) for 30 min and incubated with primary antibodies according to Table 1. Membranes were then washed with PBS-Tween (PBS-T) 0.1%, and incubated with the respective secondary antibody in agreement with Table 2. After final washing, membranes were incubated with Clarity Western ECL Substrate (BioRad) for signal detection. Densitometry analysis of protein bands was performed with ImageJ.

Table 1. Primary antibody specifications. MW: molecular weight; WB: western blot; ICC: imunocitochemistry

Target molecule	Serum	MW (KDa)	Dilutions		Supplier	Incubation conditions
			WB	ICC		
Primary antibodies						
Hsc70 (7298)	Mouse	70	1:500	-	Santa Cruz	4º overnight or 1 RT
IkBα (44D4)	Rabbit	40	1:1000	-	Cell Signalling	4ºC overnight
NF-kB p65 (C-20)	Rabbit	65	1:1000	-	Santa Cruz	4ºC overnight
Phospho-Erk	Rabbit	42/44	1:1000	-	Cell Signalling	4ºC overnight
Phospho-IkBα (S32/36)	Mouse	40	1:1000	-	Cell Signalling	4ºC overnight
Phospho-NF-kB p65 (S536) (93H1)	Rabbit	65	1:1000	-	Cell Signalling	4ºC overnight
Phospho-Src (Y416)	Rabbit	60	1:1000	-	Cell Signalling	4ºC overnight
P44/42 MAPK (Erk 1/2) (137F5)	Rabbit	42/44	1:1000	-	Cell Signalling	4ºC overnight
Src (#2109)/(#2185S)	Rabbit	60	1:1000	-	Cell Signalling	4ºC overnight
α-tubulin	Mouse	55	-	1:1000	Sigma	1h RT
Alexa Fluor 588 Phalloidin	-	-	-	1:40	Invitrogen, Molecular Probes	20 min RT

Table 2. Secondary antibody specifications. WB – Western blot; ICC – Immunocytochemistry.

Secundary antibodies					
Target		**Dilutions**		**Supllier**	**Incubation conditions**
		WB	ICC		
Mouse		1:2500	-	Santa Cruz	45 minutes RT
Rabbit		1:2500	-	GE Helathcare	45 minutes RT
Alexa 488 goat anti-mouse		-	1:500	Invitrogen, Molecular Probes	45 minutes /1h RT

RNA extraction

500 µL of TriPure Isolation Reagent (Roche) was used to extract total RNA from cells, according to manufacturer's instructions. Cells were detached, incubated for 5 min at RT to dissociate nucleoprotein complexes and 100 µL of chloroform was added to eppendorfs, which were then inverted several times during 15s. Samples were incubated for 15 min at RT and centrifuged for 15 min at 12000 *g* at 4ºC, in order to obtain separation in three phases: transparent aqueous (containing RNA), white interphase (containing DNA) and red organic (containing protein). The upper phase was transferred to a new eppendorf. 250 µL of isopropanol was added to precipitate RNA, and eppendorfs were inverted several times, gently, and incubated during 10 min at RT. After, samples were centrifuged for 10 min at 12000 *g*, 4ºC and supernatants were discarded. The pellet was resuspended in 500 µL of 75% ethanol, vortexed and centrifuged at 7500 *g* during 5 min at 4ºC. The supernatant was discarded and the excess of ethanol was removed by air drying the pellet. Subsequently, the pellet was resuspended in 20 µL RNase-free water and incubated for 1h at 4ºC. RNA concentration and purity were determined using a NanoDrop Spectrophotometer ND-1000 (Thermo Scientific). RNA samples were then stored at -80ºC until further use.

Complementary DNA (cDNA) synthesis

cDNA was synthesized using 1 µg of RNA, using SuperScript II Reverse Transcriptase kit (Invitrogen). To cDNA synthesis two independent mixes were performed. The annealing mix was composed by 1 µg RNA, random primers (3 $\mu g \mu L^{-1}$) and RNase/DNase free water, and then incubate for 10 min at 70ºC in a termocycler (MyCyder, BioRad). RT-MIX was prepared with 4 µL of 5X first-strand buffer, 2 µL of DTT (0.1M), 1µL of dNTPs (10mM, Bloron), 0.2µL of RNasin (8U, Promega), 0.5 µL of

SuperScript II RT (200 U) and 1 µL RNase/DNase free water, which was added to the annealing mix and incubated at 37ºC during 1h in a thermocycler.

Quantitative real-time PCR

The reactions of quantitative real-time PCR (qRT-PCR) were carried out using a mix constituted by 0.5uL of each cDNA sample, 4 µL water and 5 µL TaqMan Universal PCR Master Mix (Applied Biosystems).The qRT-PCR program used was composed by 2 holding stages of 50ºC for 20 s and 95ºC for 10 min followed by 40 cycles of 95ºC for 15 s and 60ºC for 1 min. Each reaction was performed in triplicate, and the PCR was run in a 7500 Real Time PCR System (Applied Biosystems). Relative mRNA expression of the target genes was normalized to the levels of the housekeeping gene using the comparative ΔΔCt method. The probes used for qRT-PCR are described in Table 3.

Table 3. TaqMan Gene Expression Assays used to assess mRNA levels for the selected genes.

Gene	TaqMan Gene Expression Assay reference
18S	Hs99999901_s1
ActB	Hs01060665_g1
CA 9	Hs00154208_m1
CCL18	Hs00268113_m1
CCR7	Hs01013469_m1
CD163	Hs00174705_m1
CD47	Hs.PT.58.45656328
CD80	Hs00175478_m1
FN1	Hs.PT.58.40005963
LDHA	Hs.PT.58.22929122
SIRP1a	Hs.PT.58.27183318
SLC2A1	Hs.PT.58.25872862
SNAI1	Hs.PT.58.2984401
SNAI2	Hs.PT.58.1772559
VM	Hs.PT.58.38906895
ZEB1	Hs.PT.58.3948500
ZEB2	Hs.PT.58.3785272

Lactate quantification assay

An enzymatic colorimetric kit (Spinreact) was used to determine lactate concentration in conditioned medium (extracellular lactate). Samples and the working reagent [50 $mmolL^{-1}$ PIPES, pH 7.5; 4$mmolL^{-1}$ 4-Chlorophenol; 800UL^{-1} Lactate oxidase; 2000UL^{-1} Peroxidase; 0.4$mmolL^{-1}$ 4-Aminophenazone] were mixed and incubated for 10

min at RT. Samples and standard absorbance (Lactate aqueous primary standard 10 mgdL^{-1}) were read at 505 nm in a multi-mode microplate reader Synergy Mix (BioTek).

Glucose quantification assay

Glucose consumption were estimated through the measurement of glucose concentration in the conditioned medium by the enzymatic colorimetric kit, Glucose Assay Kit (Roche), following the manufacturer's instructions. Briefly, samples were diluted (1:100) in Reagent Kit [200 mmolL^{-1} Phosphate buffer, pH 7.5; 0.77 mmolL^{-1} 4-aminophenazone and 11 mmolL^{-1} phenol] incubated for 20 min at RT. A standard curve was performed using 8 serial dilutions from RPMI culture medium with known glucose concentration. Glucose fluorescence intensity was detected with absorbance emission at 500nm, using a multi-mode microplate reader Synergy Mix (BioTek).

pH measurement

The pH of conditioned medium from macrophages and cancer cells in mono and co-culture, in conditions of hypoxia or normoxia, was measured using the Electro Kinetic Analyzer (EKA).

Immunocytochemistry

Immunocytochemistry staining of actin/tubulin was performed to visualize macrophages cytoskeleton. At the third day of co-culture, macrophages from mono and co-cultures in normoxic or hypoxic conditions, were fixed with 4% paraformaldehyde (PFA) for 20 min at RT. Cells were washed in PBS and then incubated with 50 mM NH_4Cl for 10 min (to block free aldehyde groups). After PBS washing, cells permeabilization was performed with 0.2% Triton X-100 for 5 min, followed by another wash with PBS. Cells were blocked for 30 min with 5% bovine serum albumin (BSA, Sigma-Aldrich). Cells were incubated with primary and secondary antibodies, according with conditions described in table I and table II. After staining, coverslips were mounted on Vectashield with DAPI (Vector Laboratories) and cells were visualized with a Zeiss Axio Imager Z1 fluorescence microscope (CarlZeiss).

Calculation of macrophage aspect ratio

ImageJ software was used to quantify the aspect ratio of actin/tubulin stained macrophages from mono and co-culture, established under hypoxia and normoxia. Aspect ratio was calculated as the ratio between the length of each cell major and minor axes. 100 cells per condition were scored.

Macrophage area measurement

Quantification of macrophage cell area was performed using Fiji software. The cell area was measured on images of actin/tubulin stained macrophages in mono- or co-cultured with RKO, and under normoxia and hypoxia conditions.

Flow Cytometry

For cell surface receptor expression analysis, macrophages were harvested with accutase (BD) for 30 min at 37ºC. To ensure that all cells were detached, cells were washed with PBS and gently scrapped and centrifuged at 1500 r.p.m. for 5 min at 4ºC and the pellet were then resuspended in flow cytometry buffer (PBS, 2%FBS, 0.01% sodium azide) and stained with anti-human CD14-allophycocyanin (APC, MEM-15), CD86- fluorescein isothlocyanate (FITC, BU63) and CD163-phycoerythrin (PE, GHI/61) (Immunotools) for 40 min at 4ºC in the dark. Additional washing steps were performed with flow cytometry buffer, and after, macrophages were fixed with 1% PFA for 20 min. Samples were filtered and then acquired with FACS Canto Flow Cytometer (BD Biosciences) using FACS Diva Software. FlowJo software was used to perform analysis of the data obtained.

In vitro wound healing assay

RKO were seeded in 24-well plates and incubated for 3 days in hypoxic or normoxic conditions at 37ºC and 5% CO_2. After 3 days, a wound was made in a confluent cell monolayers using a plastic pipette tip. The culture medium was then carefully removed, to eliminate free-floating cells and debris. Following, conditioned media from macrophages previous cultured in normoxia or hypoxia and new culture medium (1:3) was added to the cells, and the effect on wound healing was monitored. After 14 hours the plates were taken out of the incubators to be photographed using the Leica DM. Per photographed sample three random measurements were made, and samples at the initial time point (0h) were used as baseline.

Enzyme-linked Immunosorbent assay (ELISA)

Cytokines levels in conditioned media of macrophages and cancer cells from mono- and co-culture in hypoxic or normoxic were measured using ELISA TMB Development kits (Peprotech) for TNF-α, IL-6 and IL-12, following manufacturer's instructions.

Phagocytosis assay

Phagocytic activity of macrophages was evaluated using pHrodo green *Staphylococcus aureus* BioParticles conjugate (Molecular Probes). pHrodo green *Staphylococcus aureus* BioParticles conjugate was resuspended in PBS up to 1mgmL^{-1}, gently vortexed and sonicated for 5 min to obtain a homogenous dispersion. Phagocytic activity of macrophages can be measured due to the green fluorescence exhibit by the bioparticles. The green fluorescence occurs in the presence of reduced pH, which is the case of the vesicles where the particles are encapsulated after the engulfment by macrophages. Macrophages from mono- and co-culture in normoxic or hypoxic conditions ($2.4x10^5$ cells) were then incubated with $9.6x10^6$ *S.aureus* particles for 1h at 37ºC and 5% CO_2. After the incubation, macrophages were washed in PBS and fixed with 4% PFA for 20 min at room temperature. F-actin was stained with Alexa Fluor 568 Phalloidin (1:40, Invitrogen, Molecular Probes) for 20 min to cellular delimitation in acquired images, after previous permeabilization with 0.2% Triton X-100 and NH_4Cl and blocking with BSA. Lastly, coverslips were mounted on Vectashield with DAPI (for nuclei visualization) and visualized with Zeiss Axio Imager Z1 fluorescence microscope (CarlZeiss). The number of cells able to phagocyte *S.aureus* particles was determined using Fiji software. At least 200 cells per conditioned were scored.

Statistical analysis

Data analysis was performed with GraphPad Prism Software v6. Differences were tested with nonparametric test (Kruskal-Wallis test) and adjusted with the Dunn's test. Statistical significance was achieved when $P<0.05$

Results

To study the effect of the hypoxic microenvironment on the macrophage-cancer cell interplay, an indirect co-culture system was established. This system involves the indirect interaction between monocyte-derived macrophages and the human colorectal cancer cell line, RKO. We preferred to establish indirect co-cultures instead of direct ones to easily access the protein and DNA content of the distinct elements. In direct co-cultures, cell sorting would be necessary, a process that would not respect the created hypoxia conditions. The results section is subdivided in distinct parts: (I) Macrophage characterization in mono- and co-cultures with RKO cancer cells under normoxic and hypoxic conditions; (II) Characterization of macrophage and cancer cells metabolism; (III) Characterization of RKO cancer cells in mono- and co-culture with macrophages in normoxia and hypoxia.

Validation of hypoxia condition

In the first part of the study, the correct function of our experimental setup was evaluated, by assessing if cells were responding to hypoxia. Therefore, carbonic anhydrase IX (*CAIX*) mRNA expression levels were analyzed by qRT-PCR as positive control. *CAIX* was chosen as positive control since it has been described as a gene regulated by hypoxia. These analysis consistently confirmed that the *CAIX* mRNA levels increased under hypoxic conditions, both in RKO mono- and co-cultures (Fig. 6), confirming the establishment of a hypoxic microenvironment. Noteworthy, mRNA expression of *CAIX* was only evaluated in cancer cells, since macrophages do not express this gene. Nevertheless, we have then considered that if hypoxia was working on cancer cells, it was also working on macrophages, since they were subjected to the same culture conditions.

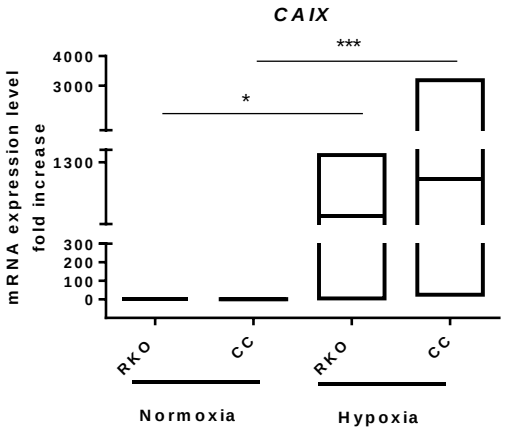


Figure 6. Anhydrase carbonic IX (*CAIX*) expression under normoxic and hypoxic conditions. *CAIX* expression analysis was used as positive control of correct function of hypoxia. Graph represent *CAIX* mRNA levels in RKO cultured alone or in co-culture with macrophages (CC), in normoxia or hypoxia. mRNA expression levels were normalized to *β-actin* expression and the results presented as fold increase relatively to RKO mono-culture in normoxia. Data represent mean values and are representative of at least three independent experiments. Kruskal-Wallis test was performed and adjust with Dunn's test; *, significantly different at P<0.05; ***, significantly different at P < 0.001.

(I) Macrophage characterization in mono- and co-cultures with RKO cancer cells under hypoxic conditions

After validating the establishment of hypoxic conditions, macrophages were characterized through different assays. Macrophage polarization, cytoskeleton organization, phagocytic capacity and signaling pathways were evaluated on macrophages co-cultured, or not, for three days, with cancer cells under normoxic or hypoxic conditions.

Naïve macrophage polarization under normoxic conditions in comparison with LPS and IL-10-stimulated macrophages

Macrophage pro- and anti-inflammatory profiles are generally associated with their ability to exert anti- or pro-tumoral activities, respectively (Mantovani *et al*. 2004). Aiming to understand if under normoxia unstimulated macrophages, herein termed as naïve, exhibit a profile anti- or pro- inflammatory, and consequently more anti- or pro-tumoral, their polarization profile was compared with one of their LPS or IL-10 stimulated counterparts, by qRT-PCR and flow cytometry analysis.

Therefore, the mRNA expression of pro- (*CD80* and C-C Chemokine Receptor 7 (*CCR7*)) and anti-inflammatory (*CD163* and *CCL18*) genes were evaluated by qRT-PCR (Fig. 7). Our results evidenced that under normoxia, naïve macrophages (MAC-N) are distinct from their LPS stimulated and IL-10 stimulated counterparts, as observed by their gene expression profile. Interestingly, LPS-stimulation induced an increase of *CD80, CCR7* and *CCL18* mRNA levels, while IL-10-stimulation enhanced

the expression of *CCL18* and *CD163*. Notably, under normoxia, the expression profile of our naïve macrophages is closer to the profile of IL-10-stimulated macrophages. Importantly, their expression profile differs from the one of LPS-stimulated macrophages, being statistically significant regarding *CCL18* expression.

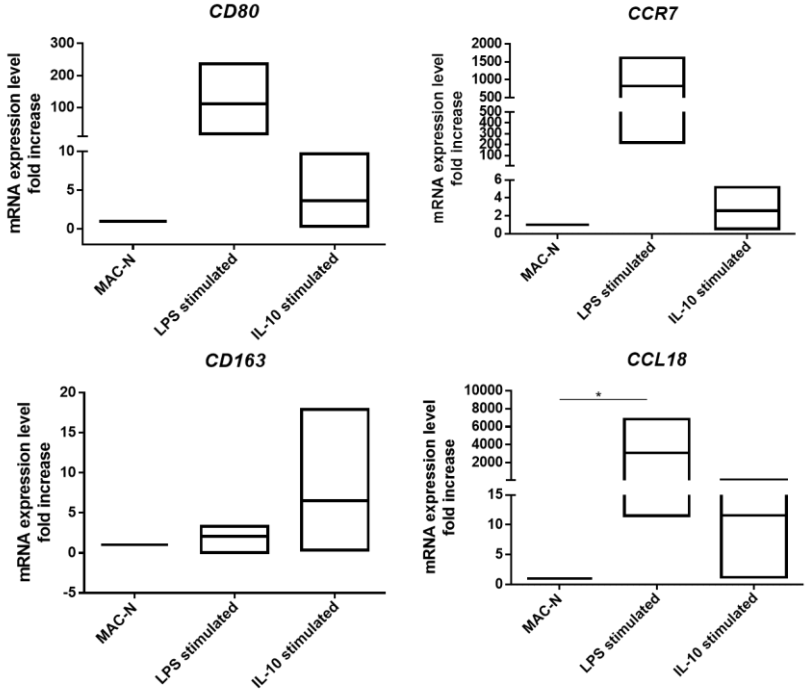
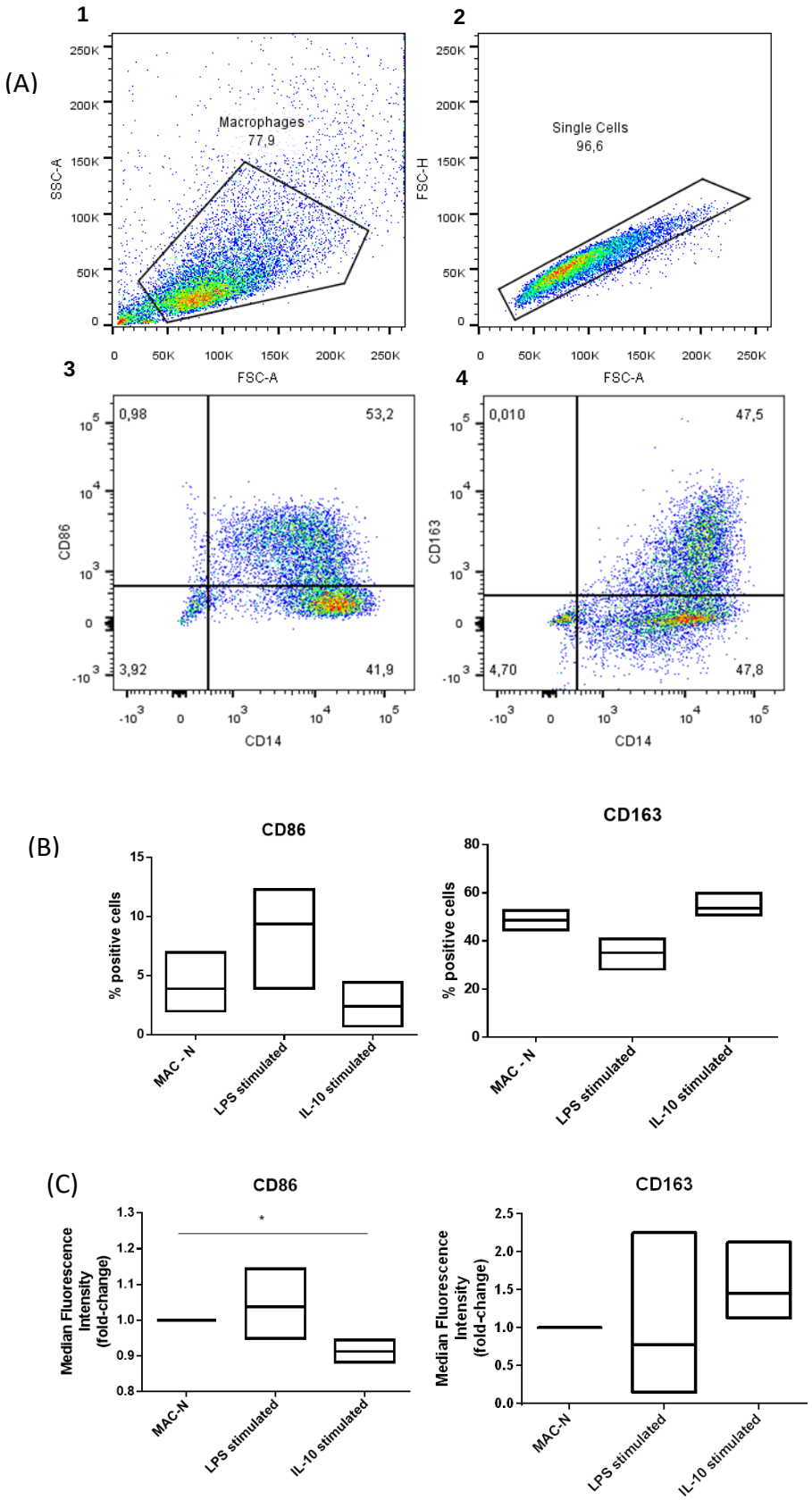


Figure 7. mRNA alterations of polarization markers in naïve macrophages exposed to normoxia, in comparison with their LPS and IL-10 counterparts. Graphs represents mRNA expression of pro- (CD80 and CCR7) and anti-inflammatory (CD163 and CCL18) macrophage markers. mRNA expression levels were normalized to *18S* expression and the results presented as fold increase relatively to macrophages in normoxia. Data represents mean values and are representative of at least three independent experiments. Kruskal-Wallis test was performed and adjusted with Dunn's test; *, significantly different at $P < 0.05$.

Flow cytometry was performed to analyze the cell surface expression of macrophage surface receptors, as the macrophage linage marker CD14, the M1-like marker CD86 and the M2-like marker CD163. The gating strategy is presented in Fig.8A, as well as the percentage of positive cells (Fig. 8B) and the median fluorescence intensity (Fig. 8C). Although some variations, over than 60% of the macrophage population maintained the expression of CD14, in all conditions. Upon LPS stimulation, the CD86 expression and the CD86 MFI increased significantly, in comparison with macrophages under normoxia. However, given the variations between distinct donors, no major differences were observed regarding CD163 expression in LPS stimulated macrophages. On the other hand, considering IL-10 stimulated macrophages no differences were found regarding CD86 or CD163 expression, in comparison with normoxic macrophages, which reinforces our qRT-PCR previous observations. To finalize these analysis, the ratio $CD14^+CD86^+/CD14^+CD163^+$ (Fig. 8D) was performed.

Interestingly, no differences were found between macrophages in normoxia or IL-10 stimulated, although, a slightly increase was observed in LPS-stimulated macrophages, indicating that this condition has more $CD86^{+}$ cells.



(D)

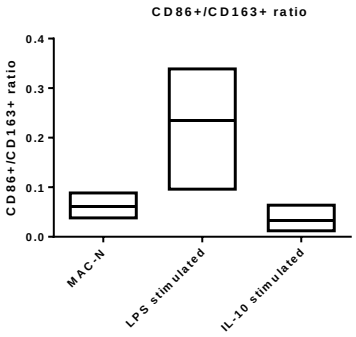


Figure 8. Differences in macrophage cell surface polarization markers under normoxia, in comparison with LPS and IL-10 stimulated macrophages. The expression of a monocyte/macrophage linage (CD14), pro- (CD86) and anti-inflammatory (CD163) macrophage markers was determined by flow cytometry in macrophages (MAC) in normoxia (N) and in macrophages LPS or IL-10 stimulated macrophages, in at least three independent experiments. (A) Pseudo-color plots exhibit the flow cytometry gating strategy created with FlowJo. 1: FSC-A/SSC-A - represents the distribution of cells in the light scatter based on cell size and granularity, respectively - to 2: FSC-A/FSC-H – represents single cells – to 3: CD14-APC/CD86-FITC positive cells or 4: CD14-APC/CD163-PE positive cells. (B) Graphs represent the percentage of $CD86^+$ and $CD163^+$ positive cells, within the $CD14^+$ cells. (C) Median fluorescence intensity from CD86 and CD163 markers was analyzed within the population of $CD14^+$ cells, and are represented in the graphs as fold change relatively to macrophages in normoxia. (D) Graph display the ratio between the $CD14^+CD86^+/CD14^+CD163^+$ cells and are representative of at least three independent experiments. Kruskal-Wallis test was performed and adjusted with Dunn's test; *, significantly different at $P < 0.05$.

Macrophage inflammatory profile under hypoxia

As we observed, naïve macrophages under normoxia seem to express a profile closer to IL-10 than to LPS-stimulated macrophages, as we have previously reported (Cardoso *et al*. 2015). The next obvious questions was to evaluate whether under hypoxia, macrophages exhibit a profile distinct to the one of macrophages in normoxia. Consequently, the mRNA expression and cell surface expression of macrophage receptors of pro- and anti-inflammatory markers were analyzed, as in the previous task. Regarding the mRNA expression, it was found that hypoxia induced a significant increase in *CCR7* expression with no alterations in the other markers expression (Fig. 9A). Interestingly, concerning the cell surface expression of these macrophage receptors, a decrease in both CD86 and CD163 markers was observed in hypoxic conditions (Fig. 9B).

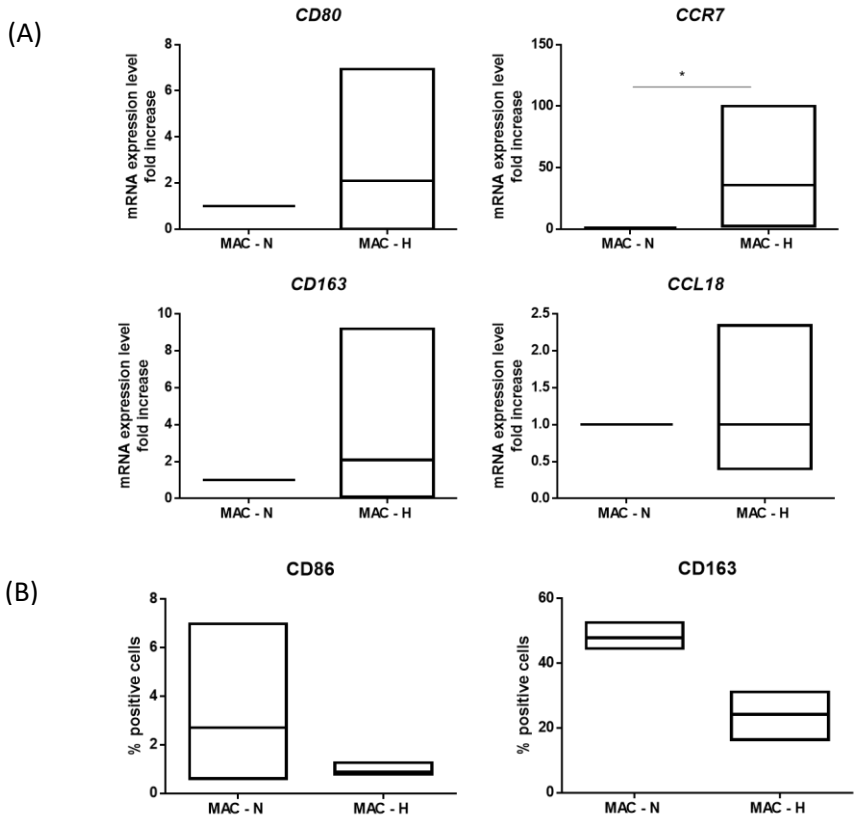


Figure 9. Differences in macrophage polarization under hypoxia. (A) mRNA expression of pro- (*CD80, CCR7*) and anti-inflammatory (*CD163, CCL18*) genes evaluation by qRT-PCR. mRNA expression levels were normalized to 18S expression and the results presented as fold increase relatively to macrophages (MAC) in normoxia (represented as dotted line). (B) The expression of a monocyte/macrophage linage marker (CD14), pro- (CD86) and anti-inflammatory (CD163) macrophage markers was determined by flow cytometry on normoxic (N) and hypoxic (H) macrophages. Graphs represent the percentage of $CD86^+$ and $CD163^+$ positive cells, within the $CD14^+$ cells. Wilcoxon matched-pairs was performed; *, significantly different at $P < 0.05$.

Macrophage inflammatory profile in co-culture, under normoxic and hypoxic conditions

Interestingly, we observed differences in the expression of polarization markers between macrophages in normoxia and hypoxia, with a clear tendency, although with reduced statistically differences. Our next question was to analyze if the presence of cancer cells, affected the macrophage polarization profile both under normoxia and hypoxia. Therefore, the same strategy and markers were used as in the previous task.

The analysis of mRNA expression of the pro-inflammatory (*CD80* and *CCR7*) and anti-inflammatory (*CD163* and *CCL18*) genes revealed that there is a tendency, although not statistically different, for the increase of pro-inflammatory markers in co-culture, with exception for *CCR7* in hypoxic conditions. Interestingly, *CCR7* levels increased when macrophages were in co-culture with RKO in normoxia, and reduced when exposed to hypoxia (Fig. 10), although without statistically significant differences. A similar tendency,

without statistically significant differences, was found for CD163 expression. Instead *CD80* expression has the tendency to increase upon co-culture, both under hypoxia or normoxia conditions. While *CCL18* expression has the tendency to enhance upon co-culture, under normoxia but not under hypoxia

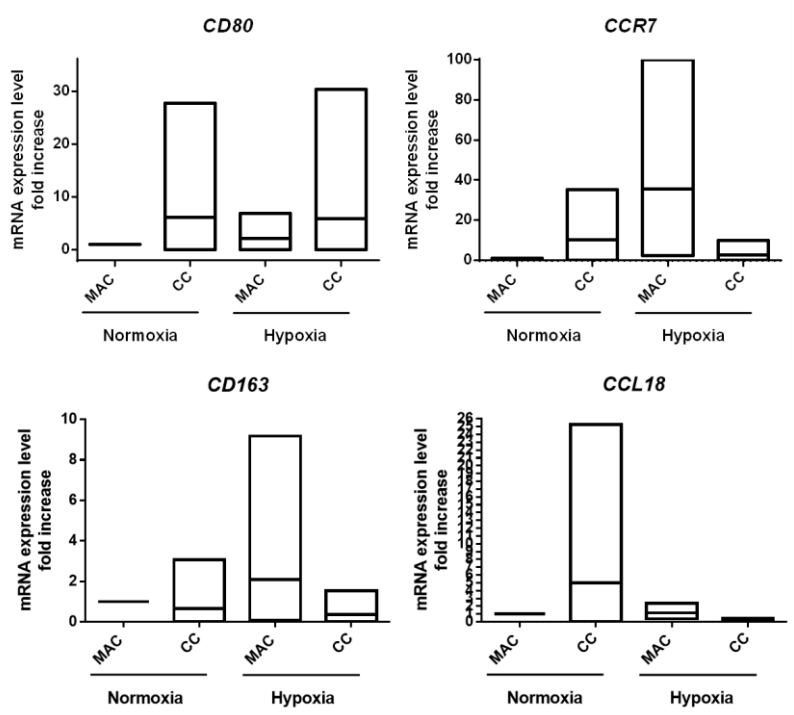
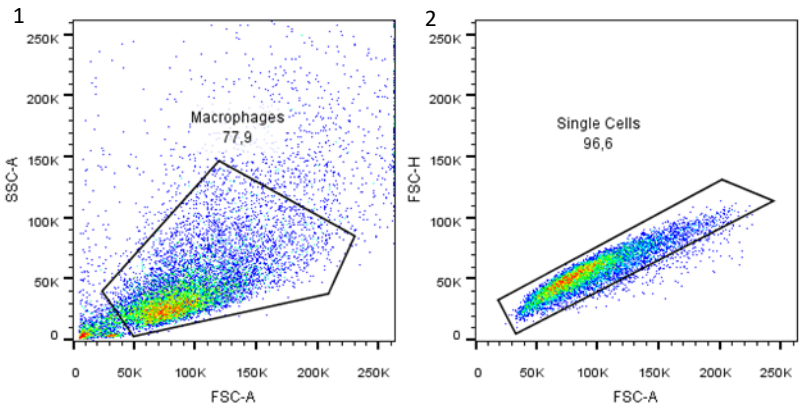


Figure 10. Macrophage inflammatory profile by qRT-PCR. Graphs represent mRNA expression of macrophages (MAC) in mono- or in co-culture (CC) with RKO, in normoxic or hypoxic conditions. mRNA expression levels were normalized to *18S* expression and the results presented as fold increase relatively to macrophages mono-culture in normoxia. Data correspond to mean values and are representative of at least three independent experiments per marker. Kruskal-Wallis test was performed and adjusted with Dunn's test.

Flow cytometry was performed in order to complement the analysis of the expression of macrophage surface receptors: a macrophage lineage (CD14), a pro-inflammatory (CD86) and an anti-inflammatory (CD163) marker. The Fig. 11A represents the gating strategy. The percentage of $CD14^+$, $CD14^+CD86^+$ or $CD14^+CD163^+$ are represented as pseudo-color plots and graphs (Fig. 11B and 11C). Additionally, for each marker individually, the median fluorescence intensity (Fig. 11D) was obtained. Over than 60% of the macrophage population maintained the expression of CD14, in all conditions. Interestingly, the percentage of $CD86^+$ cells and of CD86 MFI decreased in both hypoxic conditions, although, the differences were statistically significant only when comparing normoxic with hypoxic co-cultures. Moreover, the percentage of $CD163^+$ cells also decrease in both hypoxic conditions, and both differences were statistically significant. Nevertheless, regarding the comparison between mono- and co-cultures, no differences were found in the expression of any of these markers.

Finally, the ratio $CD14^+CD86^+/CD14^+CD163^+$ (Fig. 11E) was analyzed but no relevant statistically significant differences were observed.

(A)



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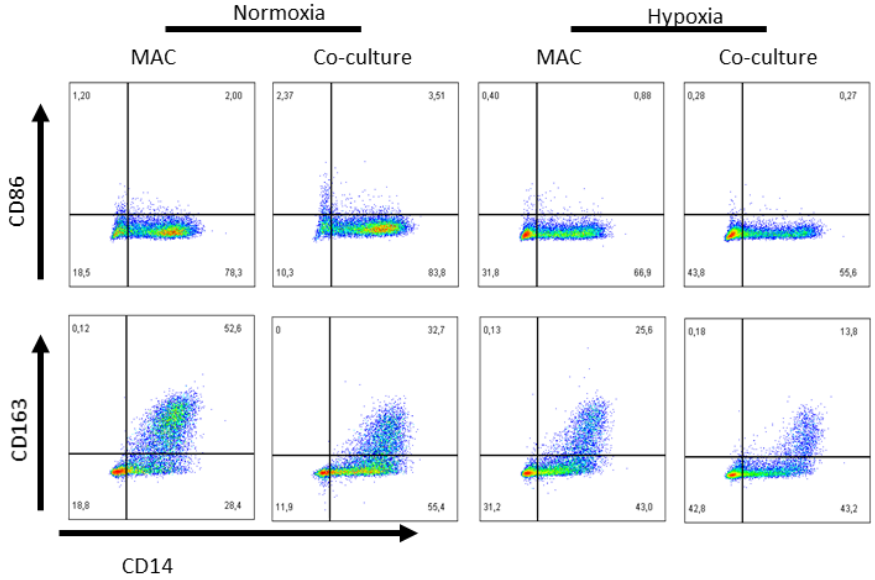

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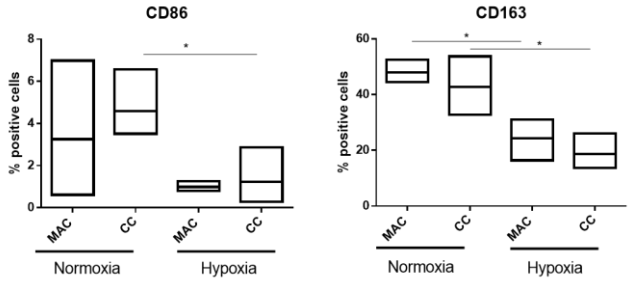

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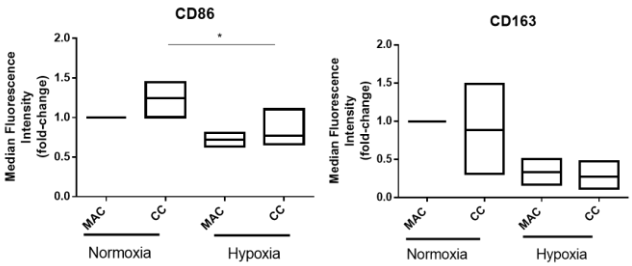

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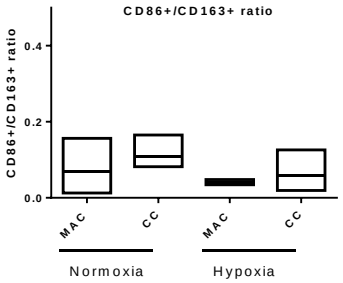


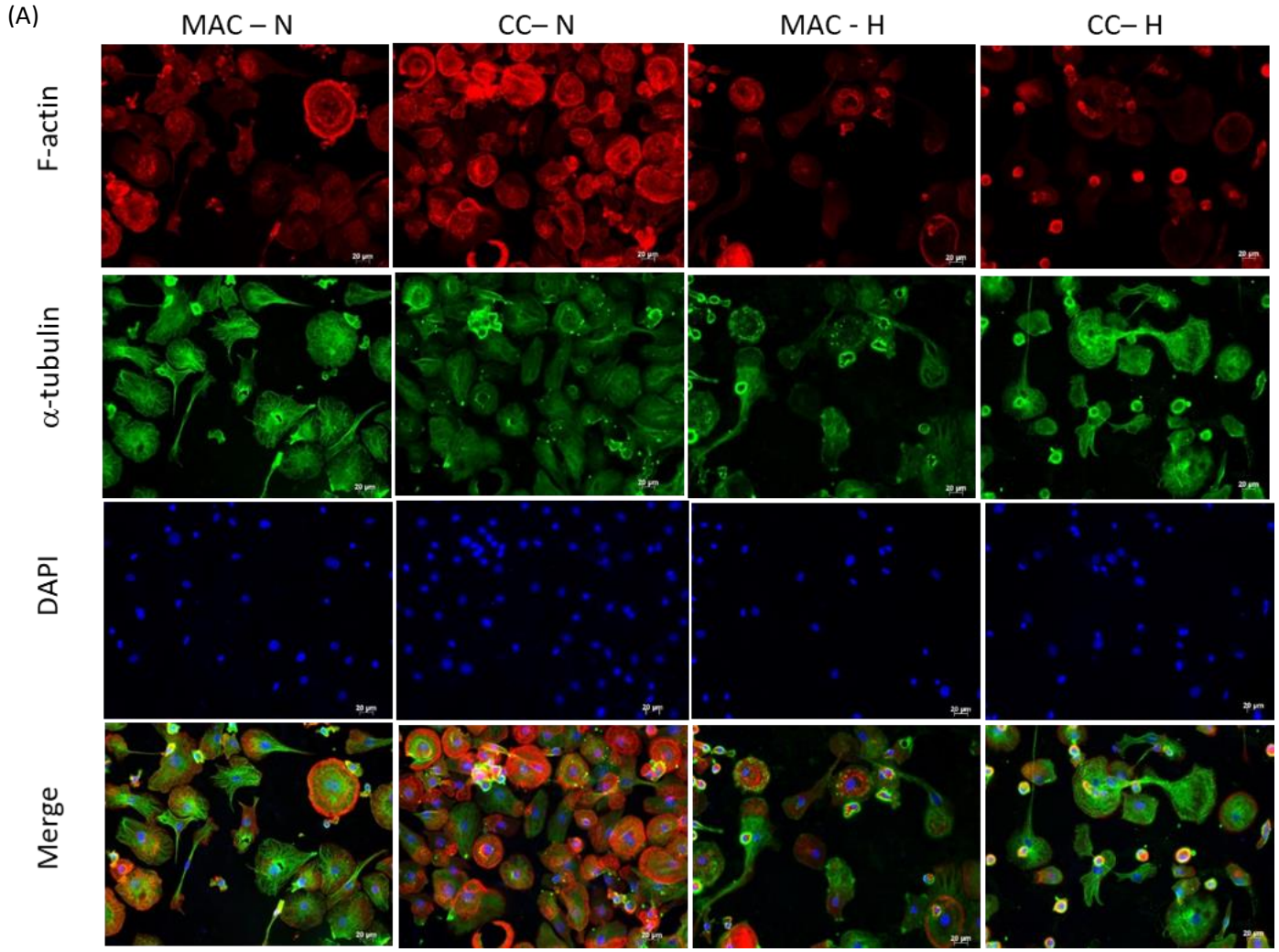
Figure 11. Macrophage inflammatory profile by flow cytometry. The expression of a monocyte/macrophage lineage marker (CD14), a pro-inflammatory (CD86) and an anti-inflammatory (CD163) macrophage marker was determined by flow cytometry in macrophages cultured alone (MAC) or with RKO (co-culture), under normoxia or hypoxia, in at least three independent experiments per marker. (A) Pseudo-color plots display the gating strategy created with Flow Jo for flow cytometry. 1: FSC-A/SSC-A - represents the distribution of cells in the light scatter based on cell size and granularity, respectively - to 2: FSC-A/FSC-H – represents single cells. (B) Pseudo-color plots with representation of CD14-APC/CD86-FITC and CD14-APC/CD163-PE double positive cells in all conditions (macrophages mono- and co-culture in normoxia and hypoxia) (C) Graphs represent the percentage of $CD86^+$ or $CD163^+$ cells, within the $CD14^+$ cells population. (D) Data represents the median fluorescence intensity of the two analyzed markers relatively to macrophages mono-culture in normoxia, represent as fold change. (E) Graph display the ratio between $CD14^+CD86^+/CD14^+CD163^+$ cells and are representative of at least three independent experiments. Kruskal-Wallis test was performed and adjusted with Dunn's test; *, significantly different at $P < 0.05$.

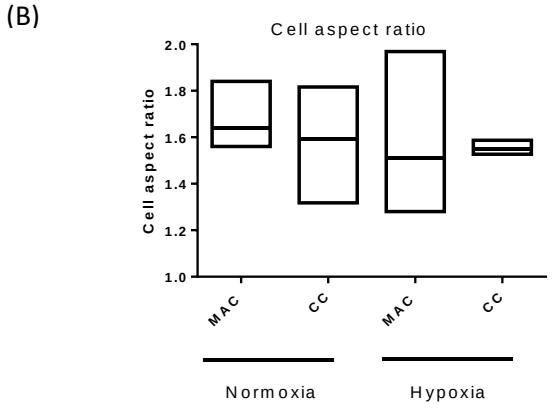
Additionally, to better characterize the macrophage polarization profile, the levels of soluble pro-inflammatory molecules as IL-6, IL-12 and TNF-α levels were also evaluated, by ELISA, in the conditioned medium from macrophages mono- and co-cultures, under normoxia and hypoxia. However, no protein expression of this cytokines was detected, which may indicate that without stimulation with LPS, these inflammatory markers are under the detection level of the assay.

Macrophage cytoskeleton organization in the presence of cancer cells under normoxia and hypoxia

The cytoskeleton organization reflects alterations in macrophage cellular organization and function, namely regarding their migratory capacity. Therefore, macrophage cytoskeleton organization was evaluated through immunocytochemistry for F-actin and α-tubulin, in mono- and co-cultures, exposed to normoxic or hypoxic conditions (Fig. 12A). In the four conditions analyzed, macrophages presented a heterogeneous morphology. However, a decrease in actin staining intensity, suggestive of reduced actin polymerization and filaments formations, was observed in hypoxic conditions. Additionally, to better characterize macrophage morphology, the cell aspect ratio, consisting in the quotient between cell major and minor axes length, was calculated (Fig. 12B). Despite the heterogeneity visualized, no major alterations were observed regarding the cell aspect ratio and cell area when macrophages were cultured alone or cultured with cancer cells, under normoxic conditions. Nevertheless, an evident

decrease on cell area and a slight decrease on cell aspect ratio were observed, although not statistically significant, when macrophages were co-culture with cancer cells, under hypoxic conditions (Fig. 12C).





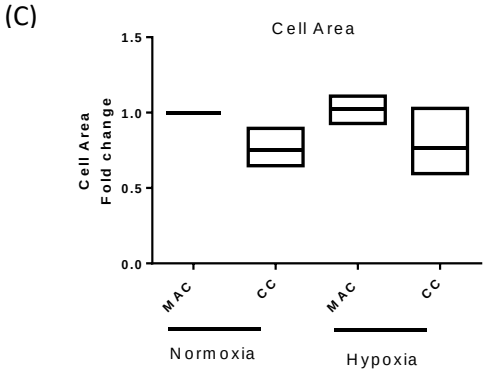


Figure 12. Macrophage cytoskeleton organization. (A) Immunocytochemistry for F-actin (red) and α-tubulin (green) was performed in macrophages (MAC) in mono- or co-culture (CC) with RKO cancer cells, under normoxic (N) or hypoxic (H) conditions. Nuclei were counterstained with DAPI (blue). (B) Morphological differences between macrophage populations were quantified by the calculation of the cell aspect ratio (quotient between cell major and minor axes) of actin/tubulin stained cells. Data represent the mean values, and are representative of at least three independent experiments (at least 100 cells/ condition). (C) Differences in the area of macrophages were quantified by the calculation of cell area of actin/tubulin stained cells. Data represent the mean values, and are representative of at least three independent experiments (at least 50 cells/ condition). Kruskal-Wallis test was performed and adjusted with Dunn's test.

Macrophage phagocytic activity in co-culture under normoxia and hypoxia

The macrophage phagocytic activity is a hallmark of pro-inflammatory macrophages, and so, differences in the macrophage phagocytic activity were evaluated in mono- and co-culture, under normoxia and hypoxia conditions. Therefore, macrophages were incubated with *Staphylococcys aureus* bioparticles for 1h, and after phagocytosis the engulfed particles exhibited green fluorescence, due to the pH reduction within the vesicles, where the particles are encapsulated (Fig. 13A). FIJI software was used to quantify the number of phagocytic cells and its mean fluorescence intensity, analyzing the photographs that were taken to macrophages. Our results revealed that co-culture with RKO cells under normoxia or hypoxia have the tendency to increase, although not statistically significant, macrophage phagocytosis (Fig. 13B). However, hypoxia results in a decrease in the macrophage phagocytic capacity, and also in the mean fluorescence intensity, in comparison to normoxia (Fig. 13C). Despite that result, mean fluorescence intensity only increase in normoxic co-culture. These results indicate that under normoxia and in co-culture with cancer cells, in addition to an increase in the number of phagocytic cells, each macrophage are able to engulf more bioparticles. Contrarily, the hypoxic co-culture showed an increase in the number of phagocytic macrophages, but each macrophage were phagocytizing less beads.

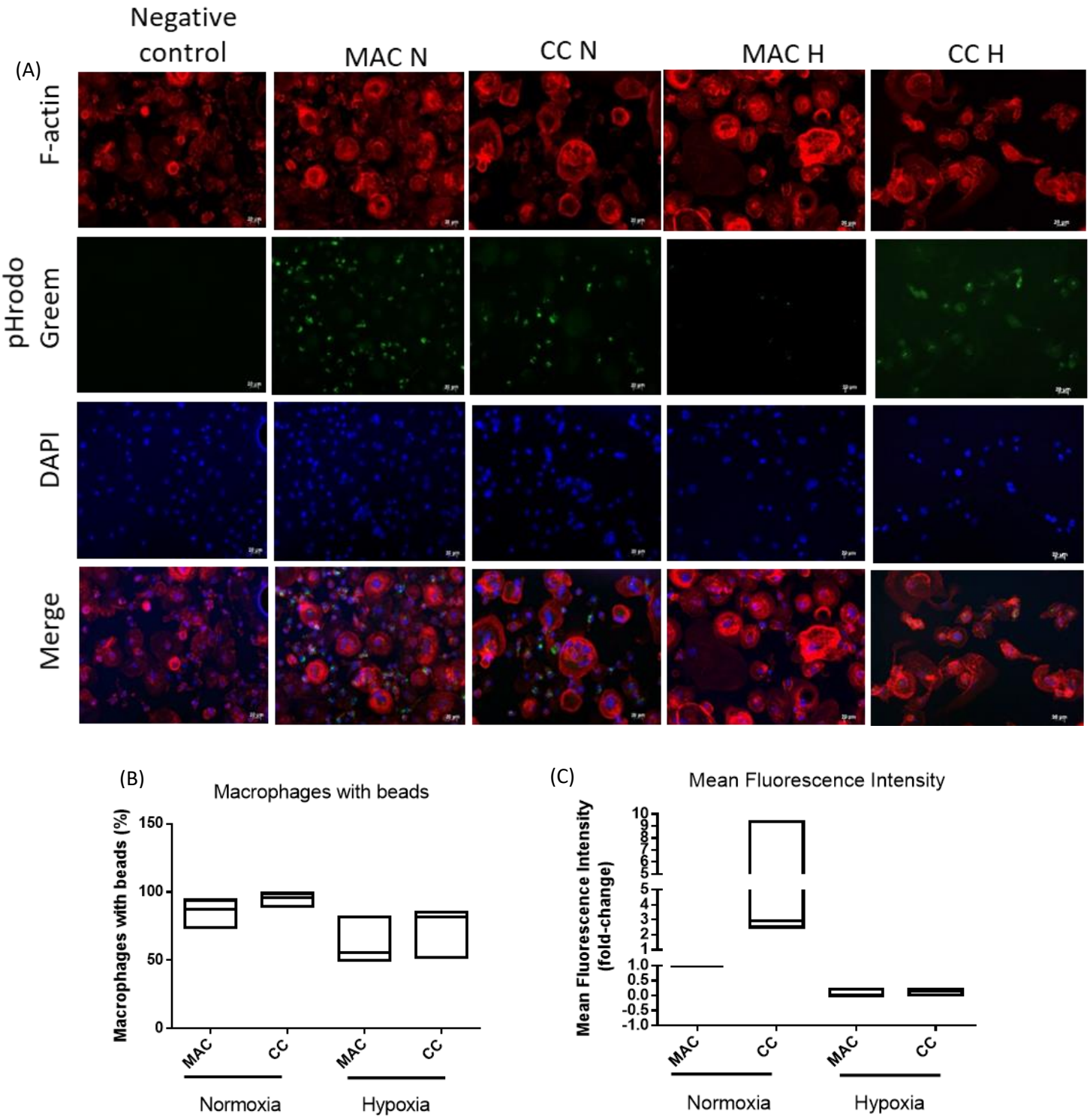


Figure 13. Macrophage phagocytic activity. (A) Macrophages cultured alone (MAC) or in co-culture (CC) with RKO, under normoxia (N) or hypoxia (H) were incubated with *S.aureus* bioparticles for 1h. F-actin was stained with Alexa Fluor 594 phalloidin. Images were taken with microscope to illustrate the phagocytosis of the FITC-labelled beads by macrophages. The negative control was performed with macrophages that did not have contact with bioparticles. (B) FIJI software was used to quantify the percentage of macrophages able to phagocyte *S.aureus* bioparticles. (C) Mean fluorescence intensity of macrophages in mono or co-culture under normoxia or hypoxia are presented as fold-change relatively to macrophages under normoxic mono-cultures. Data represent the mean values and are representative of at least three independent experiments (200 cells/ condition). Kruskal-Wallis test was performed and adjusted with Dunn's test.

In parallel, since it is described that cancer cells present “don’t eat me signals” to avoid macrophages recognition and phagocytosis, through the expression at their surface of the *CD47*, that will bind to macrophages *SIRP1α* receptor, we analyzed the mRNA levels of *SIRP1α* in macrophages and of *CD47* in cancer cells. The mRNA expression of *SIRP1α* was evaluated by qRT-PCR (Fig.14A) in macrophages in mono- or co-culture, under normoxic or hypoxic conditions. Our results revealed that macrophage mRNA expression of *SIRP1α* decreased both in hypoxic conditions, as well as in the presence of cancer cells. On its turn, the mRNA expression of *CD47* was evaluated by qRT-PCR (Fig. 14B) in RKO cancer cells in mono- or co-culture, exposed to normoxic or hypoxic conditions. Hypoxia by itself did not induce any difference on *CD47* mRNA expression. However the co-cultures with macrophages resulted in an increase of cancer cell *CD47* expression under hypoxia, while under normoxia, the presence of macrophages only induce a slight decrease in *CD47* expression.

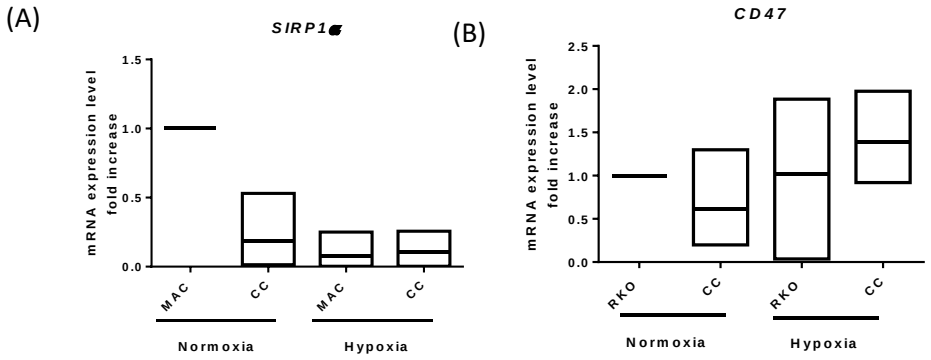


Figure 14. *SIRP1α* mRNA expression in macrophages and *CD47* mRNA in RKO cells. (A) Graph represents SIRP1α mRNA expression of macrophages in mono (MAC) or co-culture (CC) with RKO cells, under normoxia or hypoxia. mRNA expression levels were normalized to *18S* expression and the results presented as fold increase relatively to macrophages under normoxia. Data represent the mean values and are representative of at least three independent experiments. Kruskal-Wallis test was performed and adjusted with Dunn’s test. (B) Graph represents *CD47* mRNA expression of RKO cancer cells in mono (MAC) or co-culture (CC) with macrophages, under normoxia or hypoxia. mRNA expression levels were normalized to *β-actin* expression and the results presented as fold increase relatively to RKO cells under normoxia. Data represent the mean values and are representative of at least three independent experiments. Kruskal-Wallis test was performed and adjusted with Dunn’s test.

Macrophage signaling pathway analysis in the presence of cancer cells under normoxia and hypoxia

The nuclear factor kappa B (NF-κB) pathway is a signaling pathway that has been associated with cancer-related inflammation, being related with the switch from anti- to pro-inflammatory phenotype in macrophages (Hagemann *et al*. 2008). In order to explore this signaling pathway, the expression of phosphorylated and total NF-κB p65 and

IκBα was evaluated by western blot in mono- and co-cultures of macrophages, under normoxic and hypoxic conditions. Aware of the existence of consistent loading control differences, when analyzed hypoxic conditions, we have optimized the use of distinct endogenous proteins as more appropriated loading controls. The Hsc70 was the best loading control tested, after trying: β-actin, α–tubulin, cofilin and hsc90. To be more accurate, the ratio between phosphorylated protein/ total protein/ loading control (LC) was always performed and evaluated. Our results consistently revealed an increase of phosphorylated NF-κB in both co-cultures, in comparison with mono-cultures, and between monocultures of macrophages under hypoxia comparing monocultures of macrophages exposed to normoxia. Moreover, no differences were found in IκBα phosphorylation in normoxic conditions, although a slight increase was observed in IκBα phosphorylation under hypoxic co-culture, in comparison with monocultures (Fig. 15). Regarding IκBα levels, no expression was detected, and so, the levels of phosphorylated IκBα were only compared with the respective loading control.

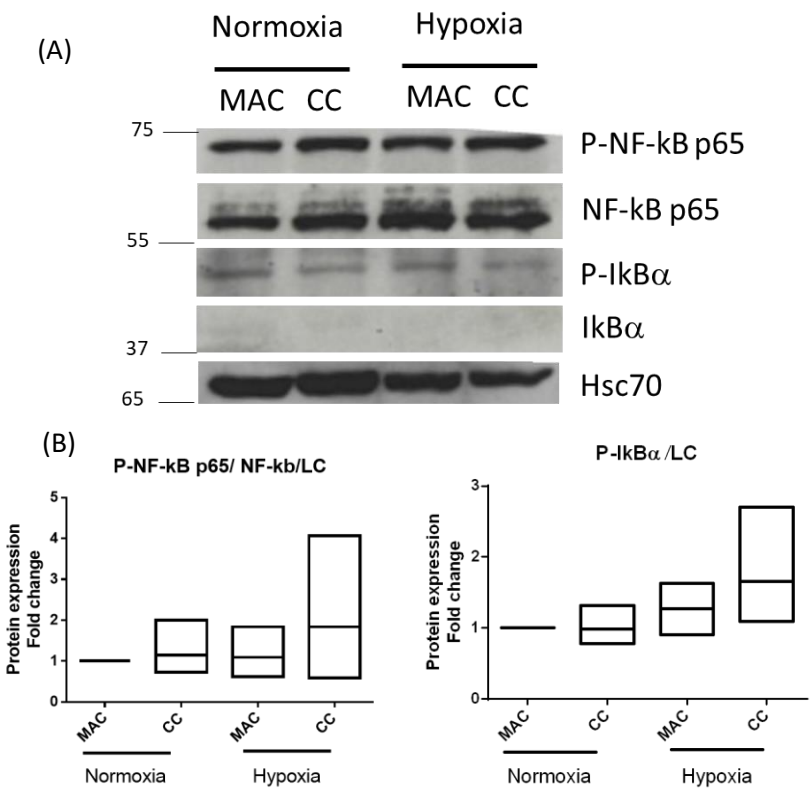


Figure 15. Analysis of macrophage signaling pathways. (A) Total and phosphorylated NF-kB and IkBα protein levels were evaluated by western blot in macrophages cultured alone (MAC) or in co-culture with RKO (CC), under normoxic or hypoxic conditions. Heat shock cognate protein 70 (Hsc70) was used as loading control (LC). (B) Protein expression was quantified using Image J and the graphs represent the fold change relatively to monocultures of macrophages under normoxia. Graphs represent the mean values and at least three independent experiments are represented. Kruskal-Wallis test was performed and adjusted with Dunn's test.

(II) Characterization of macrophage and cancer cells metabolism

The influence of hypoxia and co-culture in macrophages and cancer cell metabolism

Once hypoxia is associated with alterations in cell metabolism, and consequently different cell behavior, it is important to understand which alterations occur in macrophages and cancer cells metabolism, namely those regarding the levels of lactate production and glucose consumption. Therefore, cell metabolic activity, culture media glucose, lactate levels and pH were evaluated in both macrophages and cancer cells, when in co-culture versus monoculture, under normoxia or hypoxia.

Macrophages and cancer cell metabolic activity

Metabolic activity of macrophages and cancer cells were measured by resazurin reduction assay (Fig. 16) under normoxic and hypoxic mono- and co-cultures. Regarding macrophages, no differences were found in mono-cultures exposed to hypoxia in comparison with those exposed to normoxia (Fig. 16A). However, when in the presence of cancer cells, macrophage decreased their metabolic activity, with a statistically significant decrease under normoxia. Concerning the cancer cells metabolic activity, our results evidenced no differences, demonstrating that neither hypoxia nor macrophages affect RKO cells metabolic activity (Fig. 16B).

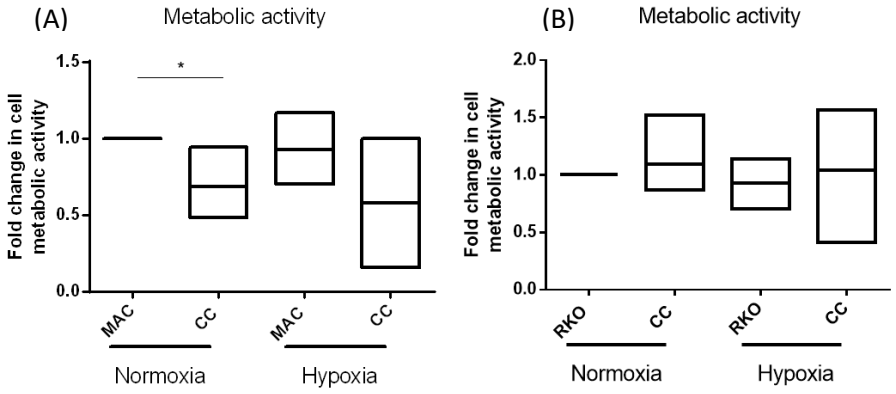


Figure 16. Macrophages and cancer cells metabolic activity. Cell metabolic activity was measured in macrophages (MAC) or cancer cells (RKO) cultured alone or in co-culture (CC) under normoxic or hypoxic conditions. Quantifications were normalized to the cell metabolic activity of mono-cultures under normoxia. Data represent the mean values and are expressed as fold change. Data are representative of at least three independent experiments. Kruskal-Wallis test was performed and adjusted with Dunn's test; *, significantly different at $P < 0.05$.

Lactate and glucose metabolism in macrophages and cancer cells

We next proceeded to evaluate the levels of lactate and glucose concentration in conditioned media from macrophages and cancer cells both mono- and co-cultures,

under hypoxia and normoxia (Fig. 17). Our results evidenced that, in both macrophages and cancer cells conditioned media, hypoxia does not alter neither lactate nor glucose concentrations (Fig. 17A and 17B). However, regarding macrophages, the presence of cancer cells induced a decreased on both lactate and glucose concentrations. On the other hand, concerning cancer cells, the co-culture with macrophages increased lactate concentration both under normoxia and hypoxia. By contrast, the glucose concentration decreased when in the presence of macrophages. Our results demonstrated that the effect of co-culture was stronger than the hypoxia effect. Moreover, the lactate-glucose ratio was evaluated and the results revealed that in all conditions, both macrophages and cancer cells, the conditioned media lactate concentration was permanently higher than the glucose concentration (Fig. 17C). Even though, in RKO conditioned media, no differences were found regarding normoxia and hypoxia mono-cultures, but a statistically significant increase in the lactate/glucose ratio was observed in co-culture conditions, both normoxia and hypoxia, comparing with mono-cultures.

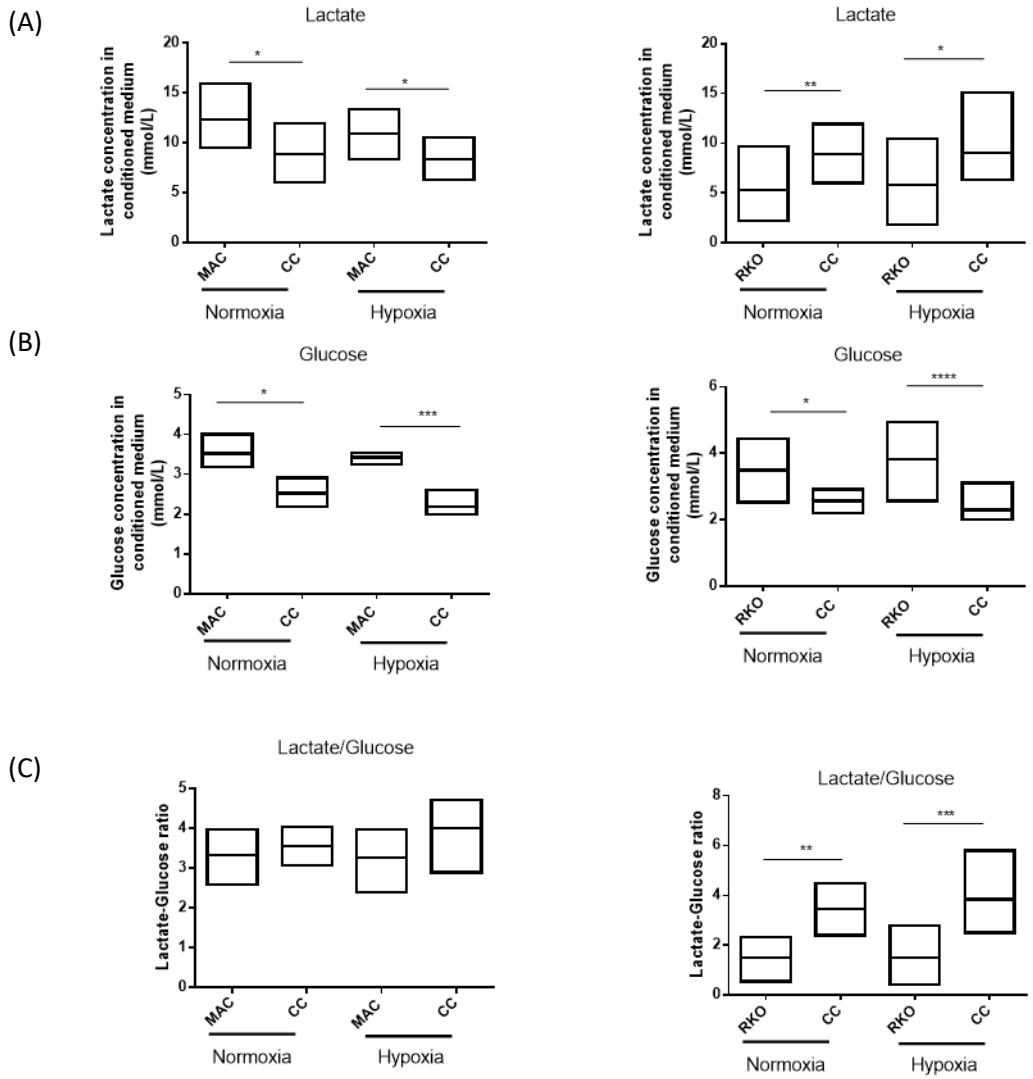


Figure 17. Lactate production and glucose consumption measurements. (A) Measurement of lactate concentration (mmol/L) was performed in conditioned media of macrophages (MAC) and cancer cells (RKO) in mono-culture or in co-culture (CC), in normoxia or hypoxia; (B) Measurement of glucose concentration (mmol/L) was performed with conditioned media of macrophages (MAC) and cancer cells (RKO) in mono-culture or in co-culture (CC), in normoxia or hypoxia; (C) Graphs are representative of lactate/glucose ratio in comparison with normoxic mono-culture. Data on the graphs represent the mean values and are representative of at least three independent experiments. Kruskal-Wallis test was performed and adjusted with Dunn's test; *, significantly different at P < 0.05; **, significantly different at P < 0.01; ***, significantly different at P < 0.001; ****, significantly different at P < 0.0001.

Once some differences were detected in the conditions analyzed, we decided to evaluate the expression levels of lactate and glucose metabolism-related genes, namely of *SLCA1* that codifies the glucose transporter (GLUT1) and *LDHA*, which expresses the lactate dehydrogenase. Analysis was performed in mono- and co-cultures of macrophages and RKO cancer cells, both under normoxia and hypoxia (Fig. 18). Our results demonstrated that hypoxia enhances the expression of both *SLCA1* and *LDHA* genes in macrophages in monocultures (Fig. 18A). However, when macrophages were co-cultured with cancer cells, a decrease of macrophages *SLCA1* expression was observed under normoxia and hypoxia conditions.

Moreover, RKO cancer cells evidenced similar results, exhibiting a slight increase in the expression of *SLCA1* and *LDHA* in mono-cultures, when under hypoxia. Instead, when co-cultured with macrophages no differences regarding the expression of both genes were observed (Fig. 18B).

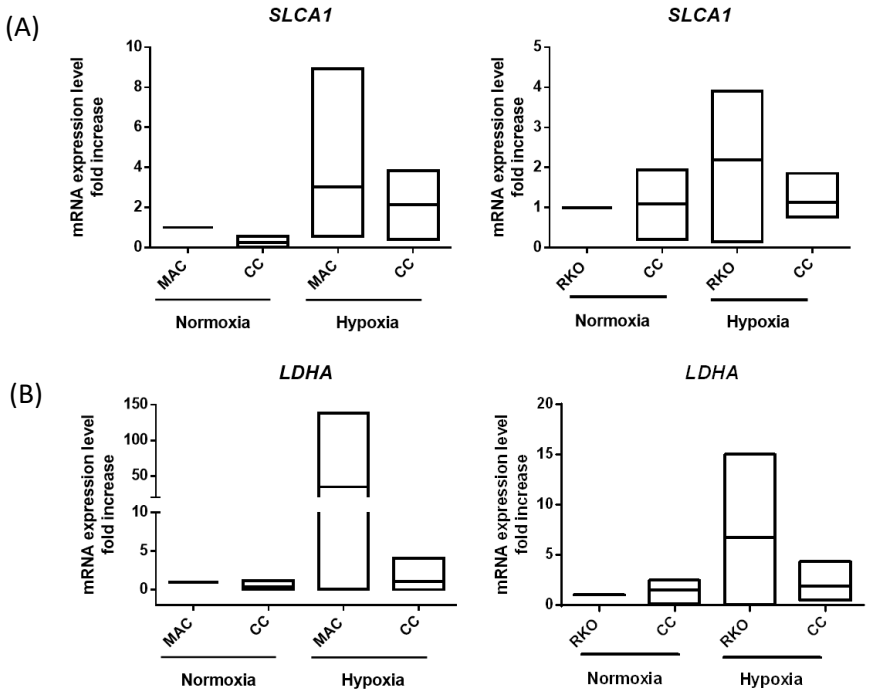


Figure 18. mRNA expression of *SLCA1* and *LDHA* in macrophages and RKO cancer cells. mRNA expression of *SLCA1* and *LDHA* was performed in macrophages (MAC) and cancer cells (RKO) mono-culture or cocultures (CC), under normoxic or hypoxic conditions. (A) mRNA expression of *SLCA1* in macrophages and RKO mono and co-cultures under normoxia and hypoxia. (B) mRNA expression of *LDHA* was performed in macrophages and RKO cancer cells cultured alone or in co-culture, in normoxia or hypoxia. mRNA expression levels from macrophages were normalized to *18S* expression and the results presented as fold increase relatively to macrophages in normoxia, while mRNA expression levels from RKO were normalized to *β-actin* expression and the results presented as fold increase relatively to RKO in normoxia. Graphs present the mean values and are representative of at least three independent experiments. Kruskal-Wallis test was performed and adjusted with Dunn's test.

pH measurements in macrophages and cancer cells

Hypoxia is frequently associated to enhanced acidosis, which emerges from the altered cellular metabolic activity (Estrella *et al*. 2013). Taking this into consideration, we decided to evaluate whether, under our experimental conditions, hypoxia and macrophage-cancer cells co-culture induce alterations on the extracellular pH levels.

Therefore, we measured the pH of conditioned media from macrophage and cancer cells mono and co-cultures, established under hypoxic and normoxic conditions. Our results revealed that, in monocultures of macrophages but not in monocultures of cancer cells, the extracellular pH decreased under hypoxia (Fig. 19). In contrast, under normoxia, cancer cells did not affect macrophage conditioned media pH but induced a slight increase under hypoxia (Fig. 19A). Interestingly, hypoxia did not affect the pH of RKO monocultures or co-cultures conditioned media but, in co-cultures, the extracellular pH decreased both under normoxic or hypoxic conditions (Fig. 19B).

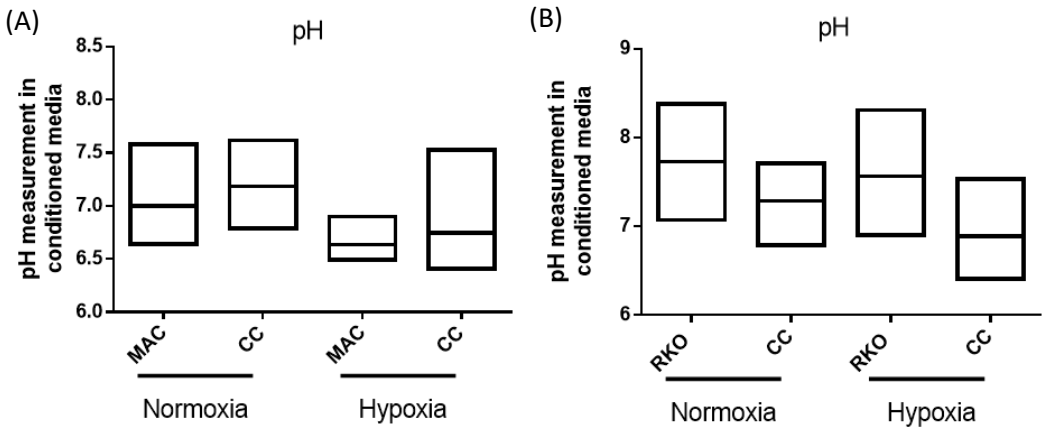


Figure 19. Evaluation of pH alterations in cancer cells and macrophage conditioned media cultured under normoxia or hypoxia. pH was measured in conditioned media from (A) macrophages (MAC) and (B) cancer cells (RKO) maintained as monocultures and cocultured (CC) under normoxia or hypoxia. Data on the graph represent the mean values and are representative of at least three independent experiments. Kruskal-Wallis test was performed and adjusted with Dunn's test.

(III) Characterization of RKO cancer cells in mono- and co-culture with macrophages in normoxia and hypoxia

After characterization of macrophages cultured in the presence of cancer cells and in a hypoxic microenvironment, we decided to focus on RKO cancer cells characterization, to understand how macrophages and hypoxia influence their invasion associated activities. Therefore, the effect of macrophage co-cultures under normoxic or hypoxic conditions on the modulation of cancer cells invasion and migration ability, gene expression, metalloproteinases production and invasion-associated signaling pathways were evaluated at the end of the experimental procedure.

Invasion capacity of RKO cancer cells

Our group has previously described that macrophages may modulate gastric and colorectal cancer cell invasion, dissecting the associated molecular mechanisms (Cardoso *et al*. 2014). Since this extensive characterization was performed under normoxic conditions, one of the objectives of this thesis was to evaluate whether the hypoxic environment change the invasion capacity of cancer cells, and also if it alters the ability of macrophages to stimulate cancer cell invasion. In order to assess if macrophages and hypoxia affect the invasion ability of cancer cells, a Matrigel invasion assay was performed, using macrophages as an invasive stimuli, under normoxia or hypoxia (Fig. 20). Our results confirmed that, in the presence of macrophages, RKO invasive capacity increased and that such effect is potentiated by hypoxia. Interestingly, we also found that hypoxia is sufficient to induce an increase in invasion, even in the absence of macrophages.

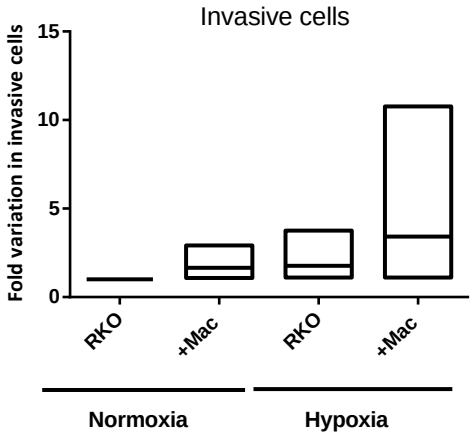


Figure 20. Effect of macrophage and hypoxia on cancer cell invasion. Invasion assays were performed using RKO cancer cells cultured alone (RKO) or in co-culture with macrophages (+Mac) in Matrigel coated transwell filters, under normoxic or hypoxic conditions. Data represent the fold variation in the number of invasive cells, and are representative of at least three independent experiments. Kruskal-Wallis test was performed and adjusted with Dunn's test.

Invasion- associated activities: cancer cell migration

Invasive cancer cells generally exhibit an enhanced ability to migrate and to cross the underlying extracellular matrix components. During this study, to evaluate the cell migration capacity of cancer cells cultured in the presence or absence of macrophages, under normoxia or hypoxia, a wound healing assay was performed. Therefore confluent cancer cell monolayers were injured and subsequently exposed, or not, to the conditioned medium of macrophages previously grown under normoxia or hypoxia (Fig. 21). Cell migration capacity was analyzed through the quantification of wound closure. Interestingly, after 14h, the controls of normoxia and hypoxia showed no differences between them. Nevertheless, while the treatment with conditioned medium derived from macrophages cultured under normoxia has the tendency to decrease cell migration, the conditioned medium derived from macrophages cultured under hypoxia have the opposite effect, suggesting to promote cancer cell migration.

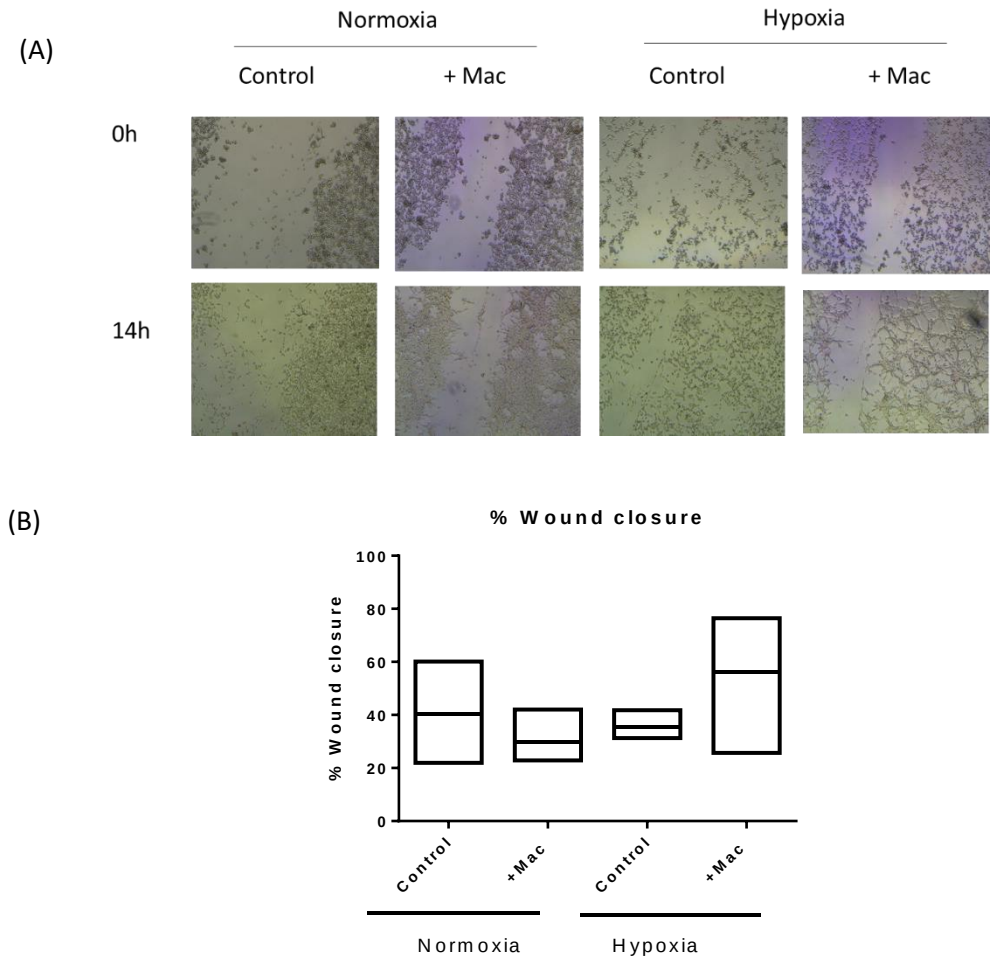


Figure 21. RKO cancer cell migration. (A) Cell migration were analyzed through wound healing assay, in RKO cancer cells cultured alone (control) or treated with conditioned medium of macrophages (+Mac), under normoxia or hypoxia. (B) Graph represents the percentage of wound closure and data is representative of at least three independent experiments, 14h after the initiation of experiment. Kruskal-Wallis test was performed and adjusted with Dunn's test.

Invasion-associated activities: cancer cell proteolytic activity

Since invasive cancer cells may exhibit enhanced proteolytic activity, which can be mediated by Matrix metalloproteases (MMPs), we decided to evaluate whether invasion-associated MMP2 and MMP9 enzymatic activity were altered by the hypoxic environment.

Therefore, conditioned media from macrophages, cancer cells and macrophage/cancer cells co-cultures were used to assess MMP-2 and -9 proteolytic activity, through gelatin zymography (Fig. 22). Our results releveled that macrophages are, indeed, the major producers of these proteolytic enzymes, and neither the presence of RKO cancer cells nor hypoxia affect macrophage pro- and active MMP9 and MMP2 gelatinolytic activity. Interestingly, hypoxia does not seem to affect RKO cancer cells MMP9 and MMP2 activities.

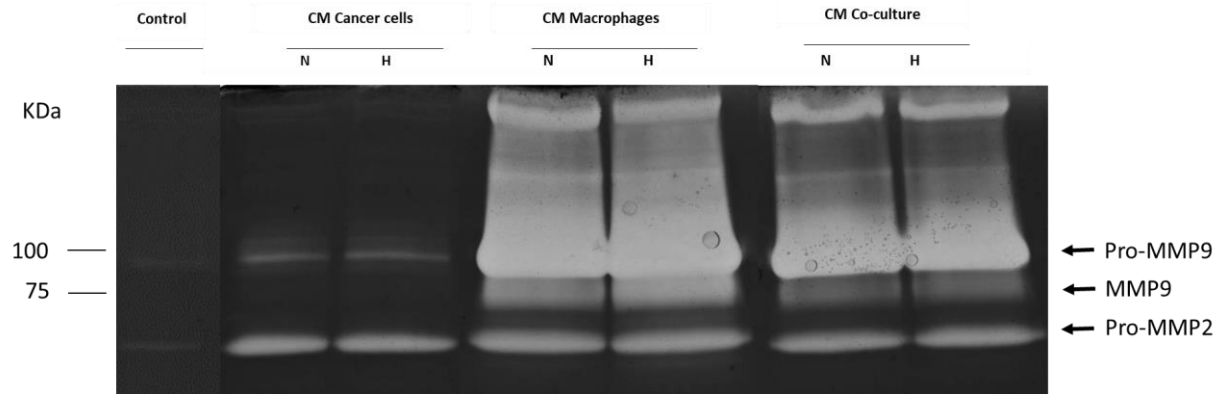


Figure 22. Effect of hypoxia on cancer cell and macrophage proteolytic activity. Activity of MMP9 and MMP2 was evaluated by gelatin zymography, using 10µg of protein from the supernatants (CM) from cancer cells and macrophages mono- and co-cultures, under normoxia (N) and hypoxia (H). RPMI was used as a negative control.

Expression of epithelial-mesenchymal transition (EMT) related genes

The acquisition of cancer cell invasive capacity is, generally associated with the acquisition of a more mesenchymal phenotype, a process named epithelial to mesenchymal transition (EMT). Differences on EMT gene profile generally dictate the acquisition of a more migratory, invasive and aggressive phenotype. Since hypoxia is considered as a tumor microenvironment element that may regulate the acquisition of mesenchymal-like characteristics, we decided to assess the expression profile of EMT associated genes (Snail 1, Snail2, Zeb1, Zeb2, FN1 and VM) in RKO cancer cells, cultured alone or in co-culture with macrophages, under normoxia or hypoxia, by qRT-PCR (Fig. 23). Our results revealed that Snail1 expression does not alter when RKO cells are exposed to hypoxia, but decreases when cancer cells are co-cultured with macrophages under normoxia, and increases when cancer cells are co-cultured with macrophages under hypoxia. Regarding Snail2 expression, we consistently observed

that this gene is not always expressed, and is never expressed under normoxic co-cultures. Similarly, Zeb2 expression does not seem to alter in response to macrophage cocultures or to hypoxia. Notably, Zeb1 expression increases with hypoxia, but macrophages seem to decrease the expression of this gene on cancer cells, both under normoxia and under hypoxia. In addition, FN1 expression did not presented any alterations with hypoxia, but when cancer cells were cultured with macrophages under normoxia, its expression seems to increase. Finally, the expression of the mesenchymal maker vimentin seems to increase when cancer cells are co-cultured with macrophages and maintained under in hypoxia. In future studies, it would be interesting to confirm whether these gene expression alterations are corroborated by protein expression differences.

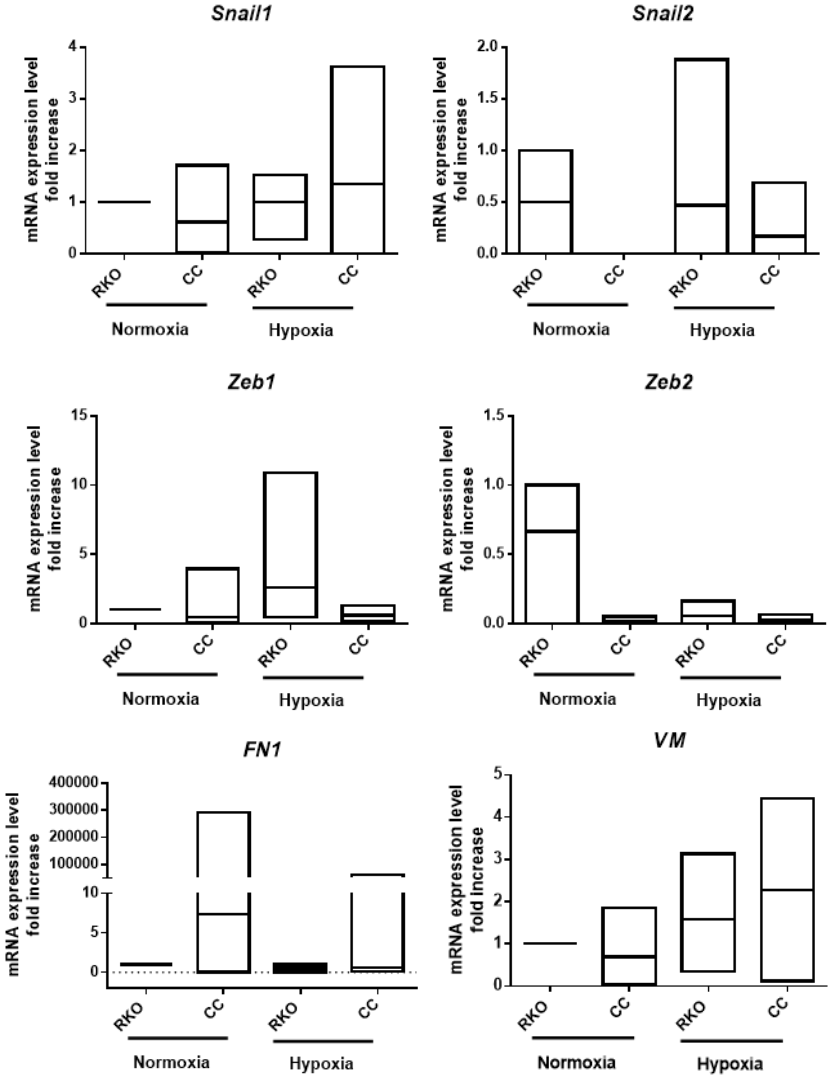


Figure 23. Analysis of epithelial-mesenchymal transition associated genes. Graphs represents mRNA expression of RKO cancer cells (RKO) in mono- or co-culture with macrophages (CC) under normoxic or hypoxia conditions. mRNA expression levels were normalized to β-actin expression and the results presented as fold increase relatively to RKO mono-culture in normoxia. Data on the graphs are representative of at least three independent experiments. Kruskal-Wallis test was performed and adjusted with Dunn's test.

Analysis of invasion-associated signaling pathways

Src and ERK signaling pathways were previously associated to cancer cell invasion phenotype (Ward *et al*. 2001; Kim *et al*. 2015) and our group has previously demonstrated that ERK and Src activation was associated with macrophage-induced gastric and colorectal cancer cell invasion (Cardoso *et al*. 2014). In order to explore these signaling pathways, the expression of total and of phosphorylated Src and ERK proteins was evaluated on cancer cells grown in monocultures, or co-cultured with macrophages, under normoxic or hypoxic conditions (Fig. 24). Interestingly, we observed that hypoxia is sufficient to induce a decrease on Src phosphorylation, and this effect seems to be significantly potentiated when cancer cells are cultured in the presence of macrophages. This effect is exclusive of co-cultures under hypoxia, once no differences were observed in normoxic co-cultures. Notably, hypoxia is also sufficient to induce an increase on cancer cell Erk phosphorylation, an increase that is significantly enhanced when cancer cells are cultured in the presence of macrophages. This effect is exclusive for hypoxic co-culture, since in normoxic co-cultures no differences were observed

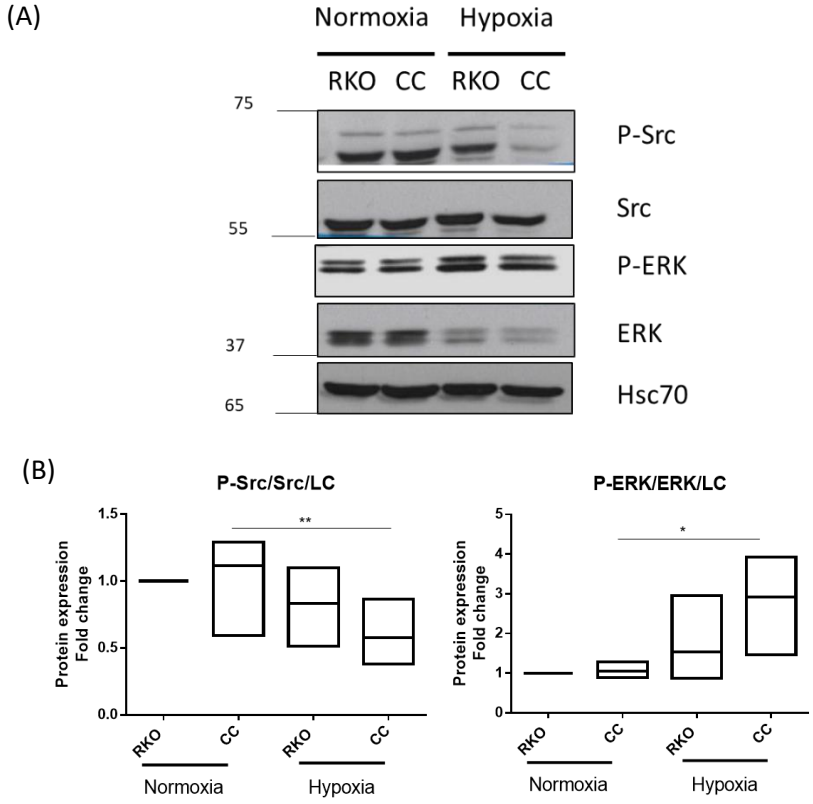


Figure 24. Analysis of invasion-associated signaling pathways. (A) Total and phosphorylated Src and ERK protein levels were evaluated by western blot RKO cultured alone (RKO) or in co-culture with macrophages (CC), under normoxic or hypoxic conditions. Heat shock cognate protein 70 (Hsc70) was used as loading control (LC). (B) Protein expression was quantified using Image J and the graphs represent the fold change relatively to RKO normoxic mono-culture. Graphs presents the mean values and represent at least three independent experiments. Kruskal-Wallis test was performed and adjusted with Dunn's test. *, significantly different at $P < 0.05$; **, significantly different at $P < 0.01$.

Discussion

The effect of hypoxia on both tumor progression (Kim *et al*. 2009; Li *et al*. 2009) and macrophage inflammatory profile (Bosco *et al*. 2006; Tripathi *et al*. 2014) has been explored. However, only few studies explored the macrophage-cancer cells crosstalk as established under hypoxic conditions (Tripathi *et al.* 2014). Interestingly, several in vitro studies have been exploring the cancer cells-macrophages crosstalk under normoxic conditions, using either direct (Shen *et al*. 2013; Zhang 2013) or indirect co-cultures (Honda *et al*. 2013; Hollmén *et al*. 2015; Wang *et al*. 2016). Others studies used conditioned medium of one population to stimulate the other population (Bögels *et al*. 2012; Wu *et al*. 2014; Lundholm *et al*. 2015). However, these approaches only allowed exploring the effect of soluble released factors, neglecting important interactions in the communication between the two cell populations. During this study, we necessarily selected indirect co-cultures to have the possibility to study the communication between the two cell populations, which is not possible when conditioned medium is used. Additionally, direct co-cultures would end with cell sorting experiments, which would no longer maintain the required hypoxic conditions. Moreover, some of the studies addressing macrophage-cancer cell crosstalk frequently use THP-1, a human monocyte cell line derived from an acute monocyte leukemia patient. Although associated with reduced variability, these cells are not representative of human monocyte-derived macrophages, and for that reason, we decided to use human peripheral-blood monocytes, as a more accurate model. In our experiments, we differentiate monocytes in the presence of M-CSF, which is a growth factor known to be involved in the recruitment of monocytes/macrophages to tissues and also, in tumors, in regulation of macrophage function *(Lin et al*. 2001; Hamilton 2008). Furthermore, in our experiment cells were exposed to hypoxia, with 1%O_2, a condition that was previously described by other authors as a hypoxic condition (Vogler *et al*. 2013; Chaturvedi *et al.* 2014). Nevertheless, in the literature, some hypoxic tumor studies were conducted using oxygen levels below 1% (Fang *et al*. 2009; Shay *et al*. 2014) or 2% (Mckeown 2014).

In order to analyze the correct function of our experimental setup, and therefore assess if cells respond to hypoxic stimuli, *CAIX* mRNA expression was analyzed in cancer cells. *CAIX* has been described as a gene regulated by hypoxia, and its expression is limitedly among normal tissues, but is widely expressed in tumors, such as colon (Frost *et al*. 2014). Its limitedly expression is the reason why we only evaluated the expression in RKO cancer cells, since no expression was detected so far in

macrophages. In our work, hypoxia increased *CAIX* expression, which confirms that our cells respond to hypoxia.

After validation of experimental conditions, we focused on the influence of hypoxia and cancer cells on macrophage functionality and inflammatory profile. Therefore, several features, such as expression of pro- and anti-inflammatory markers, cytoskeleton organization, phagocytic capacity, signaling pathways, metabolic activity, lactate and glucose metabolism and proteolysis were evaluated in macrophages cultured alone or in co-culture with cancer cells, under normoxic and hypoxic conditions. First of all, we analyzed the expression of M1-like and M2-like polarization markers in macrophages under normoxia, and in comparison with macrophages stimulated for 3 days with 10ng/mL LPS (M1-like) or with 10ng/mL IL-10 (M2-like). Our results confirmed previous reports demonstrating that normoxic naïve macrophages are more similar to IL-10 than to LPS-stimulated macrophages (Cardoso *et al*. 2015). However, their inflammatory profile is neither the same than LPS-stimulated nor than IL-10-stimulated macrophages, and therefore we considered that normoxic macrophages have a mixed phenotype, with features of both populations. Nevertheless, in hypoxia, both mono- and co-culture, showed a decrease in the pro- (CD86) and the anti-inflammatory (CD163) markers. This decrease in pro- and anti-inflammatory markers seems to indicate that macrophages in hypoxia are in the “middle” of the polarization spectrum. Contrarily, hypoxia also enhanced the expression of other pro-inflammatory marker (*CCR7*). These results of mixed phenotype are in line with divergent reports that described the upregulation and downregulation of CD86 and CD163, respectively, in primary human monocytes exposed to hypoxia (Bosco *et al*. 2006), while others referred the hypoxia as an M2-like stimuli (Leblond *et al*. 2016). To clarify this issue, the complete characterization of inflammatory profile under hypoxia should consider other technical approaches, as sequencing and proteomics, as recently showed by Court *et al. (*Court *et al*. 2017). Interestingly, the presence of cancer cells, did not result in differences in the expression of macrophages pro- or anti-inflammatory markers, contrarily to what was shown by previous studies. Several studies already demonstrated that macrophage polarization profile is influenced by cancer cells. However, this effect is dependent on macrophage origin and cancer type. For example, Bogels *et al.* demonstrated that incubation of human monocytes with colon carcinoma cell supernatants favored the production of M1-associated factors, while the incubation with breast cancer supernatants increase the expression of M2-associated factors (Bögels *et al*. 2012). Moreover, other study reported that the supernatants of two CRC cell lines, HT-29 and Colo205 induced a more pro-inflammatory (M1-like) or an anti-inflammatory (M2-like) in THP-1 cells, respectively (Wu *et al*. 2014). Although, in comparison with normoxic co-

cultures, hypoxic co-cultures also revealed a decrease in the expression of CD86 and CD163, indicating that hypoxia, either in presence or absence of cancer cells, are able to place the macrophages in a state of polarization that is more in the “middle” of the polarization spectrum. Newsworthy, it was reported by Torroella-Kouri *et al.* that mice mammary tumors peritoneal macrophages are a less polarized population, with lower expression of several macrophage markers (Torroella-kouri *et al*. 2009).

Macrophage cytoskeleton organization is very susceptible to alterations in the environment (Mcwhorter *et al.* 2013). For this reason, we evaluated macrophages cytoskeleton organization, cell aspect ratio and cell area of macrophages, when cultured alone or in co-cultured with cancer cells, under normoxic or hypoxic conditions. In our work hypoxia decreased macrophages aspect ratio. Modifications in cytoskeleton organization has been described in the literature, and our group and other authors have previously confirmed that LPS-stimulated macrophages (M1-like) exhibit a more elongated shape than the IL-10-stimulated ones (M2-like) (Cardoso *et al*. 2015; Ploeger *et al*. 2013). Therefore, our results suggest that despite the variability observed in macrophage population, under hypoxic conditions, macrophages display a more rounded shape (cell aspect ratio more close to 1), which is associated with a M2-like profile. However, when analyzing the cell area, despite no alterations found in hypoxia, in the presence of cancer cells the macrophage cell area seem to reduce, both in normoxia and hypoxia. These results can indicate that macrophages in the presence of cancer cells are less differentiated, since it was previously described that when macrophages became differentiated they became bigger (Zhang *et al*. 2012).

Furthermore, macrophage phagocytic ability was evaluated in macrophages in mono- or co-cultures, under normoxia or hypoxia, since they are considered professional phagocytes. We demonstrated that the phagocytic ability of macrophages decreases in hypoxia, while it increases in both normoxic and hypoxic co-cultures in comparison with mono-culture. Additionally, we also observed a decrease in phagocytic ability of macrophages co-culture in hypoxia, in comparison with normoxia. These results suggest that hypoxia is affecting phagocytic ability, which is support by a study evidencing that the phagocytic ability of alveolar macrophages decreases when exposed to hypoxia (Leeper-woodford & Mills 1992). However, the reports of hypoxia effect on macrophage phagocytic ability have some conflicting results. A more recent study reported that hypoxia enhanced phagocytosis rate of particles *in vitro* in RAW264.7 murine macrophages and primary peritoneal macrophages (Anand *et al*. 2007). These results could indicate that macrophages origin and also differences in oxygen percentage influence differently the macrophage phagocytic capacity.

Regarding the effect of cancer cells on macrophage phagocytic capacity, the results described in the literature are inconsistent, once one study described that phagocytic index of macrophages isolated from breast cancer patients showed a decrease (Arsenijevi *et al.* 2005), while another reported an increase in phagocytic capacity of THP-1 cells cultured with supernatants from CRC cell lines (Wu *et al*. 2014). These results might indicate that the impact on macrophage phagocytic ability is dependent on cancer type and macrophage origin. Moreover, it is important to refer that our phagocytosis experiment consists in the engulfment of bacteria-conjugated particles, and that M1-like macrophages are more related with microorganism killing, while M2-like macrophages are more linked with phagocytosis of cell debris (Mantovani *et al*. 2002), and it was also described that M2-like macrophages are less capable of bacterial phagocytosis (Varin *et al*. 2010). Altogether, these results may indicate that, under hypoxia, macrophages lose an important M1-associated feature, while in the presence of cancer cells this activity is restored. Additionally, we evaluated in RKO cancer cells the mRNA expression of *CD47*, and in macrophages the mRNA expression of *SIRP1α*. *CD47* has been associated with phagocytosis inhibition in macrophages (Navarro-alvarez & Yang 2011), based on its interaction with signal regulatory protein alpha (SIRP1α), which is expressed on macrophage surface (Zhang *et al.* 2013). Interestingly, a decrease were observed in *SIRP1α* expression in both co-culture and hypoxia, although when in normoxic co-culture, a decrease was observed in *CD47* expression. The expression of *CD47* can be related with macrophages phagocytosis activity, since in normoxic co-culture we observed an increase in phagocytosis, which could be associated with macrophage response to a decrease in *CD47* expression. Notably, the differences in phagocytosis in hypoxia cannot be explained by differences in *CD47* expression.

NF-kB transcription factors are important for cell survival and response under diverse environmental signals (e.g. hypoxia), but are also considered major regulators of inflammation processes, being related with switch from anti- to pro-inflammatory phenotype in macrophages (Hagemann *et al*. 2008). In unstimulated cells, NF-kB proteins are mainly localized in the cytoplasm and associated with inhibitory proteins, such as IkBα. Several stimuli are able to activate the NF-kB pathway, therefore increasing phosphorylation of IkBα proteins, which results in its ubiquitination and proteosomal degradation, leading to NF-kB release, allowing their nuclear translocation and further activate gene expression (Yamamoto & Gaynor 2004). Our results showed an increase in NF-kB phosphorylation in both co-cultures, in comparison with mono-cultures. Moreover, no differences were found in IkBα phosphorylation in normoxic

conditions, although a slight increase was observed in IkBα phosphorylation in hypoxic co-culture, in comparison with mono-culture. Due to problems with the loading control, even though several molecules were tested, they always showed differences between normoxic and hypoxic conditions, therefore only mono- and co-cultures were compared. Given this, our results seems to indicate that in hypoxia, the presence of cancer cells activate NF-kB pathway, allowing NF-kB translocation to the nucleus which results in activation of gene expression and then cytokines release, that could be related with tumor development (Hagemann *et al*. 2009).

Macrophage mitochondrial function was evaluated by the resazurin reduction assay. Our results demonstrated that hypoxia does not alter macrophage mitochondrial function, although the presence of cancer cells, either in normoxia or hypoxia decrease their metabolic activity, suggesting that cancer cells have the ability to change macrophage metabolism. Although, Bogels *et al*. demonstrated that supernatants of colon and breast cancer cells increase macrophage metabolic activity (Bögels *et al*. 2012), which could mean that different conditions lead to different alterations in macrophage metabolism. Additionally, we also evaluated RKO metabolic activity, and our results evidenced that neither hypoxia nor macrophages affects RKO mitochondrial function. Furthermore, we also analyzed glucose and lactate, in both macrophage and RKO mono- and co-cultures, under normoxia and hypoxia. First, we quantified the lactate and glucose concentration in conditioned media, and we found that hypoxia did not alter neither lactate nor glucose concentration, neither in macrophages nor RKO mono-cultures. It also revealed that basal concentration of lactate in conditioned medium from macrophages is higher than in conditioned medium from RKO. Therefore, when in co-culture the concentration of lactate decrease in comparison with macrophages mono-culture, but increase comparing with RKO mono-culture. Regarding glucose concentration, it decreases in co-cultures, both in comparison with macrophage and RKO mono-cultures, which can be mainly explained by the fact that two different cell populations are consuming more glucose than only one population, and therefore the concentration in the conditioned medium decreased. The differences in metabolic activity of macrophages, as well as glucose and lactate concentration in co-culture might be linked to alterations in macrophage polarization, since M1-like and M2-like exhibit metabolic profiles very distinct. M1-like macrophages are more linked to aerobic glycolysis, characterized by increase in glucose uptake, as well as enhanced conversion of pyruvate to lactate (O'Neill & Pearce 2015), while in M2-like macrophages the majority of energy is obtained from fatty acid oxidation and oxidative metabolism, and Krebs cycle overcomes glycolysis (Galván-Peña & O'Neill 2014).

Additionally, we also analyzed mRNA expression of *SLCA1*, that encodes the glucose transporter 1 (GLUT1), a membrane-bound protein responsible for glucose uptake, and *LDHA*, which encodes the lactate dehydrogenase A, an enzyme responsible for the conversion of pyruvate to lactate. Interestingly, despite no alterations were observed in the glucose and lactate concentration, an increase was observed in both *SLCA1* and *LDHA* in hypoxic mono-culture, both in macrophages and RKO. This increase in *SLCA1* in hypoxic macrophages are in accordance with the previously described by Burke *et al*, where it shows that *SLCA1* is upregulated by hypoxia (Burke *et al*. 2003). Moreover, other authors also reported the upregulation of both *SLCA1* and *LDHA* in hypoxia (Semenza 2011). On the other hand, no alterations were observed in co-cultures gene expression, in contrast to the differences obtained in glucose and lactate concentration, which could indicate that other glucose transporter are activated in these conditions.

Once differences in metabolism are associated with differences in pH, to finalize the metabolic characterization, extracellular pH was measured in conditioned medium from macrophages and RKO mono- and co-cultures, under normoxia and hypoxia. This analysis revealed that hypoxia induce a slight decrease in macrophages pH in mono-cultures. However, comparing co-cultures with mono-cultures, while an increase is observed in case of macrophages, in RKO there is a decrease. These alterations in co-cultures are concomitant with the alterations observed in lactate concentration, and are similar to the results described in previous studies (Hashim *et al*. 2011; Calorini *et al.* 2012).

After characterization of hypoxia and co-culture influence on macrophages, we focused on the alterations that both hypoxia and macrophages can induce on RKO cancer cells. As previously described by our group, macrophages induces an increase in RKO invasion ability (Cardoso *et al*. 2014). Therefore, we evaluated the invasion ability of RKO cells that were cultured, during 72h in mono- and co-culture with macrophages, under normoxia and hypoxia. Our results revealed that hypoxia by itself is capable to increase RKO invasion ability. Although, when in the presence of macrophages in hypoxia, this increase is potentiated. Notably, Shen *et al*. has described similar results obtained with *in vitro* co-culture of gastric cancer and macrophages (Shen *et al*. 2013). Additionally, cancer cell migration was also analyzed and it was observed that in the presence of macrophage conditioned medium, in hypoxia, the cancer cells migration increased, while in normoxia decrease. Using mouse macrophages and a breast cancer lines, Green *et al* verified an increase in cancer cell migration (Green *et al*. 2009), in normoxic conditions, while our results show an increase only in hypoxia, which might indicate that different cancer cell lines respond differently to macrophages.

MMP activity is an important factor for macrophage-mediated cancer cell invasion, migration and motility that was previously described by our group (Cardoso *et al*. 2015). In our study, MMP9 and MMP2 activity was evaluated in conditioned medium of macrophages and RKO cultured alone or in co-culture, under normoxia or hypoxia. Between normoxic and hypoxic mono-cultures of RKO no differences were found neither in MMP9 nor in MMP2 activity. However, in comparison with mono-cultures, in co-cultures an increase were observed, which is due to the macrophage contribution, since they are the major producers in this co-culture. Moreover, no differences were observed in MMP2 activity, neither between macrophage nor co-culture, both in normoxia and hypoxia. Although, no differences were found in MMP9 activity between macrophages under normoxic and hypoxic conditions, neither in comparison with co-cultures. Interestingly, our team has previously described that cancer cells are able to modify the MMP9 activity, showing an increase in MMP9 activated, but no alterations in the proMMP9. Moreover, our team also reported that MMP9 production is higher in IL-10 stimulated macrophages (M2-like) (Cardoso *et al*. 2015), therefore our results suggests that in co-culture macrophages profile is not altered.

Epithelial-mesenchymal transition (EMT) has been described as an important feature for cancer cell motility and invasiveness (Christiansen & Rajasekaran 2006; Fenouille *et al*. 2012), and therefore we evaluate the expression of some EMT-related genes. *Snail1, Snail2, Zeb1, Zeb2, FN1* and *VM* expression was analyzed in RKO mono- and co-culture, under normoxia or hypoxia. Our results revealed that the *Snail1* expression was not altered when cells are exposed to hypoxia, but decrease when co-cultured with macrophages in normoxia, and increase in co-culture in hypoxia. Regarding *Snail2* expression, this gene is not always expressed in the control, and is never expressed in normoxic co-cultures. The *Zeb1* expression increase with hypoxia, although its expression decrease when in co-culture with macrophages, both in normoxia and hypoxia, while *Zeb2* gene have low levels of expression. *FN1* expression did not presented any alterations with hypoxia, but when co-culture with macrophages in normoxia it expression increase. Lastly, the *VM* expression increase in hypoxia and in hypoxic co-culture, although it decrease in normoxic co-culture. A previous study has described an increase in *Snail* and *VM* expression, both mesenchymal markers, when co-cultured pancreatic cancer cells with M2-polarized macrophages (Liu *et al*. 2013). Taken together, these results may indicate that our macrophages are more M1-like macrophages, so do not produce M2-like factors that are related with increase in *Snail* and *VM*. Moreover, it has been described that hypoxia upregulates the expression of *Zeb1* and *Zeb2* (Bock *et al*. 2011), although, we only observed an increase in *Zeb1*, which could indicate that they are differently regulated.

Src and Erk pathways has been extensively studied and its activation are associated with cancer cell invasion phenotype (Ward *et al*. 2001; Huang *et al*. 2004; Guarino 2010; Cardoso *et al*. 2014; Kim *et al*. 2015). In our work, no differences were found in Src phosphorylation in normoxic co-culture, although in hypoxia a decrease was observed, in both mono- and co-cultures. Moreover, the hypoxic co-culture presented a statistically significant decrease in Src phosphorylation compared with normoxic co-culture. No alterations were observed in ERK phosphorylation in normoxic co-culture, however in hypoxia it was found an increase. Furthermore, hypoxic co-culture presented a statistically significant increase in ERK phosphorylation when compared with normoxic co-culture. Interestingly, it has been previously described by our group that macrophages, in normoxia, induces colorectal cancer ERK1/2 phosphorylation, although they also described an increase in Src phosphorylation, after 60min of treatment with conditioned medium from macrophages (Cardoso *et al*. 2014). Our results differ from the ones previously described, which could suggest that different culture conditions can influence the macrophages-colorectal cancer crosstalk. Moreover, the opposite effect of macrophages in hypoxia, in Src and ERK phosphorylation might indicate that these two molecules are differently regulated upstream. Curiously, Liu *et al*. described that hypoxia are able to activate ERK1/2 phosphorylation *in vitro* and *in vivo* in gastric cancer cells (Liu *et al*. 2010). Moreover, Src activation was also reported to occur in response to hypoxia (Hasan *et al*. 2012). Taken together, these results indicate that both hypoxia and the presence of macrophages, together, have an important role in Src and ERK signaling pathway.

Altogether, our results demonstrate that macrophages and cancer cells establish a complex crosstalk (Appendix 1 and 2), and that hypoxia influence this crosstalk, making it even more challenging its full comprehension.

Conclusions and future perspectives

Our study was based on the establishment of an indirect co-culture system between macrophages and colorectal cancer cells, exposed to normoxic or hypoxic conditions, and revealed that both cells influence on each other, additionally to the effect of oxygen tension.

Macrophage characterization revealed that either cancer cells or hypoxia, individually or together, induced a mixed phenotype in macrophages, with both M1 and M2-like characteristics. We have analyzed important features of macrophages, specifically their inflammatory profile, cytoskeleton organization, phagocytosis, NF-kB signaling pathway, metabolic activity (including lactate and glucose) and proteolytic activity when they are in the presence or absence of cancer cells, under a normoxic or hypoxic microenvironment. Hypoxia is more powerful than the presence of cancer cells in regarding the expression of polarization markers, the phagocytic activity and the expression of metabolism-related genes. We also observed that, in contrast, the co-culture of cancer cells with macrophages is powerful than hypoxia in the modulation of macrophage cell area, metabolic activity and lactate/glucose metabolism. However the combination of hypoxia and co-cultures potentiate the phosphorylation of NF-kB and of IkBα. To better characterize the effect of cancer cells and hypoxia on macrophage functionality and polarization profile, numerous pro- and anti-inflammatory molecules and cytokines need to be analyzed through flow cytometry, qRT-PCR and ELISA, making the use of arrays, potential interesting tools to use in the future. Additionally, a more detailed study on macrophage metabolism will be also important, analyzing the expression of more metabolism-related genes and proteins, namely other glucose transporters.

Concerning the effect of hypoxia and macrophages on cancer cells, we also analyzed important characteristics of RKO cancer cells related with their function and invasion activity. RKO characterization revealed that several mechanisms are involved in cancer cell response to macrophages and hypoxia. We found in the conditions analyzed that hypoxia has a more powerful effect than the co-culture regarding the expression of metabolism-related genes and of some EMT-associated genes, as well as the Src and ERK phosphorylation. We also observed that, in contrast, the co-culture with macrophages is powerful than hypoxia in modulating *CD47* expression, lactate/ glucose metabolism and the expression of some EMT-associated genes. However the combination of hypoxia and co-culture with macrophages potentiates the effect on cancer cell *CD47* expression, invasion and migration ability and phosphorylation of Src and ERK proteins. These results pointed to a need to have in consideration both the

presence of macrophages/cancer cells and hypoxia, when choosing the treatment options. To get into more detail on the impact of macrophages and hypoxia on cancer cells invasion-associated activities, it would be important to analyze more signaling pathways that could be involved in tumor progression. These results should be also corroborated in other cell lines.

Generally, the model used in this work of an *in vitro* indirect co-culture system is a simple model to investigate the interaction between macrophages and cancer cells, under normoxic or hypoxic environment. It is an important tool to understand how cancer cells and macrophages connect to each other and how they respond to hypoxia, allowing characterizing the mechanisms involved in this response.

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Appendix

Appendix 1 – Summary of the macrophage results obtained in this work. (*) - Significantly different

	CC Normoxia	MAC Hypoxia	CC Hypoxia
MAC Normoxia	⬆ CCR7 = CD80, CD163, CCL18 = CD86, CD163 = Cell aspect ratio ⬇ Cell Area ⬆ Phagocytosis ⬆ P-NF-kB = P-IkBa ⬇ SIRP1a ⬇ Metabolic activity (*) ⬇ Lactate and glucose concentration (*) = SLCA1, LDHA = pH = MMP9 and MMP2 proteolytic activity	⬆ CCR7 = CD80, CD163, CCL18 ⬇ CD86, CD163 = Cell aspect ratio = Cell Area ⬇ Phagocytosis ⬇ SIRP1α = Metabolic activity = Lactate and glucose concentration ⬆ SLCA1, LDHA ⬇ pH = MMP9 proteolytic activity = MMP2 proteolytic activity	
CC Normoxia			⬇ CCR7 = CD80, CD163, CCL18 ⬇ CD86, CD163 (*) = Cell aspect ratio, = Cell Area ⬇ Phagocytosis = SIRP1a = Metabolic activity = Lactate and glucose concentration = SLCA1, LDHA = pH = MMP9 proteolytic activity = MMP2 proteolytic activity
MAC Hypoxia			⬇ CCR7 = CD80, CD163, CCL18 = CD86, CD163 = Cell aspect ratio ⬇ Cell area ⬆ Phagocytosis ⬆ P-NF-kB, P-IkBα = SIRP1a ⬇ Metabolic activity ⬇ Lactate and glucose concentration (*) = SLCA1 ⬇ LDHA = pH = MMP9 proteolytic activity = MMP2 proteolytic activity

Appendix 2 – Summary of the RKO cancer cells results obtained in this work. (*) - Significantly different

	CC Normoxia	RKO Hypoxia	CC Hypoxia
RKO Normoxia	= Metabolic activity ⬆ Lactate concentration (*) ⬇ Glucose concentration (*) = SLCA1, LDHA ⬇ pH ⬆ Invasion ability = Wound healing ⬆ MMP9 proteolytic activity = MMP2 proteolytic activity ⬇ Snail1, Snail2, Zeb2, VM = Zeb1 ⬆ FN1 = P-Src, P-ERK ⬇ CD47	= Metabolic activity = Lactate and glucose concentration ⬆ SLCA1, LDHA ⬇ pH ⬆ Invasion ability = Wound healing = MMP9, MMP2 proteolytic activity = Snail1, Snail2, FN1 ⬇ Zeb2 ⬆ Zeb1, VM = P-Src, P-ERK = CD47	
CC Normoxia			= Metabolic activity = Lactate and glucose concentration = SLCA1, LDHA = pH ⬆ Invasion ability ⬆ Wound healing = MMP9 proteolytic activity = MMP2 proteolytic activity = Snail1, Zeb1, Zeb2 ⬆ Snail2, VM ⬇ FN1 ⬇ P-Src (*) ⬆ P-ERK (*) ⬆ CD47
RKO Hypoxia			= Metabolic activity ⬆ Lactate concentration ⬇ Glucose concentration (*) ⬇ SLCA1, LDHA ⬇ pH ⬆ Invasion ability ⬆ Wound healing ⬆ MMP9 proteolytic activity = MMP2 proteolytic activity ⬆ Snail1, VM = Zeb1, FN1 ⬇ Snail2,Zeb2 ⬇ P-Src ⬆ P-ERK ⬆ CD47